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Desgroseillers et al.

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(54) **METALLOPROTEASES OF THE NEPRILYSIN FAMILY**

(56) **References Cited**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

WO WO 99/53077 10/1999

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(Continued)

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(2), (4) Date: **Aug. 21, 2001**

(57) **ABSTRACT**

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In this paper, we describe RT-PCR strategies that allowed us to identify and clone members of the NEP-like family. Degenerate oligonucleotide primers corresponding to consensus sequences located on either side of the HEXXH consensus sequence for zincins were designed and used in RT-PCR with mouse and human testis cDNAs. DNA fragments with lengths expected from the sequence of this class of enzymes were obtained. These DNA fragments were cloned and sequenced. Using this PCR strategy and the PCR fragments as probes to screen cDNA libraries, three zincin-like peptidases were identified in addition of known members of the family. The cDNA sequences allowed to derive specific probes for Northern and in situ hybridization, and probe human chromosomes to localize the gene and establish potential links to genetic diseases. Furthermore, these cDNA sequences were used to produce recombinant fusion proteins in *Escherichia coli* in order to raise specific antibodies. Finally, the cDNA sequences were cloned in mammalian expression vectors and transfected in various mammalian cell lines to produce active recombinant enzymes suitable for testing specific inhibitors.

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C12N 9/48 (2006.01)
C12N 1/20 (2006.01)
C12N 15/00 (2006.01)
C12Q 1/37 (2006.01)
C07H 21/04 (2006.01)

(52) **U.S. Cl.** **435/212; 435/4; 435/6; 435/252.3; 435/320.1; 435/69.1; 435/71.1; 435/440; 435/24; 536/23.2; 536/23.5**

(58) **Field of Classification Search** **435/183, 435/252.3, 320.1, 4, 71.1, 212, 440, 6, 69.1; 536/23.2, 23.1, 23.5; 530/350**

See application file for complete search history.

6 Claims, 22 Drawing Sheets

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10      20      30
NEP1-HU  NGR.....SESONDITDINT...PKKXKORRYLEI.....LSLVMLLL
PEX-HUN  NER.....KDG...SSEVET...GKFNHSGIRLALVV.....FVGSITVLSG
KEL1-HU  MEHGDSSEEPREASQAGMGTLAGSSTPEERLPVSGSPKAV...ARRVTRILL..
ECE1-HU  NSTYKRAFLDSESLVDSISSEGVAVPRLQVHHSFRRGQRVARIQVSKRLVLLVLLA
consens  K      T      P      L

40      50      60      70      80      90
NEP1-HU  TIIAVTHIALSA..TYDD...GICNSDCKIAARLIQNDATTEICTDFEYAGQWLFK
PEX-HUN  TIIELVSCQLLQIQAQ...EYCLKPECIKAAALISKVVLSVDFCNHFEACDQWLN
KEL1-HU  GLLALCTFVLLFTYFQHCGERFCSTVCLDARHILASGQTVAPCFEYFPEQ...RA
ECE1-HU  AGLVACLAALGQ...QVTRSPFVCLSEACVSTSELESDPTVDPCHDFEYVAGQWIKK
consens  L      L      C      C      L      V      PC      DFF      AGQW

100     110     120     130     140     150
NEP1-HU  WHFPTSSRHVGNFDLIDBLELVLRVQLQEP...KEDIVAVGAKARALYRGINESRIDS
PEX-HUN  HPIEDMFSVGVYENLRHVQLKRELEKISRRRTALQKAKLYSSQNHAKLEKA
KEL1-HU  KETHNS...FQLATKHWRLRLLEVD...NSHNFSGSEENAFQFVNSCMETLIDAR
ECE1-HU  HNFVDDSRHGTFSNLMERNQALIKHLENS..TA..SVSEDRNANQVYRAGHRETRLEL
consens  N      F      G      F      L      LK      LE      A      KA      Y      S      O      N   AIE

160     170     180     190     200
NEP1-HU  GGEFLALSLDFPI..YKPF...VATEHEDQVYDAS..WIAEKATLQVLSKQVSRVILNLYNTD
PEX-HUN  DAKVLLHLLHSDFRHFVLESTGPEQWNSZKTLQLLACFRGQVSSVSLVLYSD
KEL1-HU  QTDFLQVIEEL.....GDWISGKNTSLNPN...RTLRMLSGYGHFFFRVYLGPN
ECE1-HU  RANFLMELIEEL.....GGNHITDQAKDFQ...DELQVWVWVYVTSFVSVVSD
consens  FL      G      W      F      TL      Y      ?      YV      D

220     230     240     250     260
NEP1-HU  DQHSVHVHIIHQPRGLPSR..DYECTGIKXACTAYDMSVARIHQEELPI..DE
PEX-HUN  DRASVHILLDQCLSLAVREDYLDHSTENSVYDALLYFVQSTAVL.....LGA..NS
KEL1-HU  FASPTFVVICDQFEPVPLAGDQEDF..YADIFRE..VLTPEMLQCTL.....LQD..CF
ECE1-HU  SRSVSHVCIQVDSGLGFRDYVLRNTEKRVLTG..VLMHNVOLGK.....LQGDDE
consens  K      S      VI      DQ      L      LP      R      DV      X      Y      H      L      LG      D

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1 10 20 30
 NEP1-HU MGK.....SESQMDITDINT..PKPKKKQRWTPLEI.....SLSVLVLLL
 * * * * *
 PEX-HUM MEA.....ETG....SSVET..GKKANRGTRIALVV.....FVGGTLVLG
 ** * * * * *
 KELL-HU MEGGDQSEEEPRERSQAGGMGTLWSQESTPEERLPVEGSRPWAV....ARRVLTAILL.
 * * * * *
 ECEI-HU MSTYKRATLDEEDLVDSLSEGDAYPNGLQVNFHSPRSGQRCWAARTQVEKRLVVLVLLA
 consens M T P L

40 50 60 70 80 90
 NEP1-HU TIIAVTMIALYA.TYDD...GICKSSDCIKSAARLEQNMDATTEPCTDFEKEYACGGWLKR
 ** * * * * *
 PEX-HUM TILFLVSQGLLSLQAKQ...EYCLKPECIEAAAAILSKVNLSVDPCDNFFRFACDGIWISN
 * * * * *
 KELL-HU .GLLLCFSVLLFYNFQNGFRPCETSVCLDLDRHYLASGNTSVAPCTDFESEACG...RA
 ** * * * * *
 ECEI-HU AGLVACLAALGI.QYQTRSPSVCLSEACVSVTSSILSSMDPTVDPCHDFFSYACGGWIK
 consens L L C C L V PC DFF ACGGW

100 110 120 130 140 150
 NEP1-HU NVIPETSSRYGNFDILRDELEVVLKDVLPQEP..KTEDIVAVQKAKALYRSCINESAIDSR
 * * * * *
 PEX-HUM NPIPEDMPSYGVYPWLRHNVDLKLEKELLEKSISRRTDEAIQKAKILYSSCMNEKAIEKA
 * * * * *
 KELL-HU KETNNS.....FQELATKNKNRLRRILEVQ.NSWHPGSGEEKAFQFYNSCMDTLAIEAA
 * * * * *
 ECEI-HU NPVPDGHRSRWGTFSHLWEHNQAIKHLLENS.TA.SVSEAEKKAQVYYRACMNETRIEEL
 consens N P G F L LK LE A KA Y SCMNE AIE

160 170 180 190 200
 NEP1-HU GGEPLLKLLPDI.YGWP..VATENWEQKYGAS.WTAEKAIQQLNSKYGKKVLINLFGVTD
 *** * * * *
 PEX-HUM DAKPLLHILRHSPFRWPVLESNIGPEGVWSEKFSLLQTLATFRGQYSNSVFIIRLYVSPD
 ** * * * * *
 KELL-HU GTGPLRQVIEEL.....GGWRISGKWTSLNEN..RTLRLMSQYGHFFFRAYLGP
 ** * * * * *
 ECEI-HU RAKPLMELIERL.....GGWNITGPWAKDNEQ..DTLQVWTAHYRTSPFFSVYVSAD
 consens PL G W F TL Y F YV D

220 230 240 250 260
 NEP1-HU DKNSVNHVIHIDQPRGLPSR.DYYECTGIYKEACTAYVDFMISVARLIRQEERLPI.DE
 ** * * * * *
 PEX-HUM DKASNEHILKLDQATLSLAVREDYLDNSTEAKSYRDALYKFMVDTAVL.....LGA.NS
 * * * * *
 KELL-HU PASPHTPVIOIQIDQPEFDVPLKQDQEQKI.YAQIFRE.YLTYLNQLGTL.....LGG.DP
 *** ** * * * * *
 ECEI-HU SKMSNSNVIQVDQSGGLPSRDYYLNKTENEKVLTG.YLNYMVQLGKL.....LGGGDE
 consens K S VI DQ L LP R DY K Y M L LG D

115 - 1

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270      280      290      300      310      320
NEP1-HU  NQLALEMNVMELEKEIANATAKPEDRNDPMLLYNKMTLAQIQNNFSLEINGKPFWSLNF
           + * ** ** * * ** * **
PEX-HUM  SRAEHDMKSVLRLEIKIAEIMI PHENRTSEAMY.NKMNISELSAMIP.....QFDWLGY
           * * * * * * * * * * *
KELL-HU  SKVQEHSSLSISITSRLFQFLRPLEQRRRAQGKLEQMVITIDQLKEMAP.....AIDWLSL
           * * * * * * * * * * *
ECE1-HU  EAIRPQMQQILDFETALANITIPQEKRRDEELIYHKVTAELQTLAP.....AINWLPF
consens  M     E     A     P E R     K T L P     WL

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330      340      350      360      370      380
NEP1-HU  TNEIMSTVNI SITNEEDVVVYA...PEYLTCLKPILTKYSARDLQNLMSWRFEIMDLVSS
           + * * * * * * * * * *
PEX-HUM  IKKVIDTRLYPHLKDISPSENVVVRVPQYFKDLFRILGSEKKTIANYLWVRMVYSRIPN
           * * * * * * * * * *
KELL-HU  LQATFTPMSLSPSQSLVVHDVEYL...KNMSQOLVEMLLKQRDELQSHMILGLVVTLSPA
           * * * * * * * * * *
ECE1-HU  LNTIFYPVEINESEPIVVYDKEYL...EQISTLINT...TDRCLLNNYMIWNLVKRKTSSF
consens           V                 L             L N M W V

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390      400      410      420      430
NEP1-HU  LSRTYKESR...NAFRKALYGT.TSETATWRRRCANYVNGNMENAVGRLYVEAAFAGESK
           *** * ** * * * * * * * * *
PEX-HUM  LSRRFQYRW...LEFSRVIQGT.TLLPQWDKCVNFIESALPYVVGKMFVDVYFQEDKK
           * ** * * * * * * * * *
KELL-HU  LDSQFQEARRKLKLSQKLRELTEQPPMPARPRWMKCVETGTFFFEPTLAALFVREAFGPSTR
           ** ** * * * * * * * * *
ECE1-HU  LDQRFQDA...DEKEMEVMYGTCKTCLPRWKFCVSDTENNLGEALGPMFVKATFAEDSK
consens  L  FQ           F     GT     P W CV           G  FV  F  K

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440      450      460      470      480      490
NEP1-HU  HVVEDLIAQIREVFIQTLD.DLTWMDAETKRAEEKALAIKERIGYPDDIVSNDNKLNNE
           * * * * * * * * * * * * * * *
PEX-HUM  EMMEELVEGVRWAFIDMLEKENEWMDAGTKRKAKEKARAVLAKVGYPE.FIMNDTHVNEDE
           * * * * * * * * * * * * * *
KELL-HU  SAAMKLFTAIRDALITRLR.NLPWMNEETQNAQDKVAQLQVEMGASE.WALKPELARQE
           * * * * * * * * * * * * * *
ECE1-HU  SIATEIILEIKKAFEEELS.TLKWMDDEETKSAKEKADAIYNMIGYPN.FIMDPKELDKV
consens  L  IR AFI  L  L WMD ET  A EKA A  GYP
              (4)  (IA/B)

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500      510      520      530      540      550
NEP1-HU  YLELNYKEDEYFENIIQNLKFSQSKQLKKLREKVDKDEWISGAAVVNAFYSSGRNQIVFV
           * * * * * * * * * * * * * * *
PEX-HUM  LKAIKFSEADYFGNVLQTRKYLAQSDFFWLRKAVPKTEWFTNPTTVNAFYSASTNQIRFV
           * * * * * * * * * * * * * *
KELL-HU  YND.IQLGSSFLQSVLSCVRSRLRARIQVSEFLQHPQHRWKVSPWVDNAYYSVSDHVIVFV
           ** * * * * * * * * * * *
ECE1-HU  ENDYTAVPDLYFENAMRFFNFVSWRVTADQLRKAPNRDQWSMTPPMVNAFYSPKNEIVFV
           (2A/B)
consens  YF N           LR           W  P  VNA YS  N IVFV

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FIG. 1 (cont'd)

	560	570	580	590	600	610
NEP1-HU	AGILQPPFFSAQQ.SNSLNYGGIGMVGHEITHGFDDNGRNFNKDGDLDVWWTQQSASNF					
	* * * *	* * * *	* * * *	* * * *	* * * *	* * * *
PEX-HUM	AGELQKPPFFWGTEYPRSLSYGAIGVIVGHEFTHGFDNNGRKYDKNGNLDPWWSTESEEFK					
	* * * *	* * * *	* * * *	* * * *	* * * *	* * * *
KELL-HU	AGLLQPPFFHPGY.PRAVNFGAAGSIMAHELLHIFYQL...LLPGGCL...ACDNHAL					
	* * * *	* * * *	* * * *	* * * *	* * * *	* * * *
ECE1-HU	AGILQAPFYTRSS.PKALNFGGIGVVVGHETHAFDDQGREYDKDGNLRPWWKNSSEAF					
consens	AG LQ PFF	P LN G IG	GHE TH FD GR	K G L WW	S F	

	620	630	640	650	660	670
NEP1-HU	KEQSQCMVYQYGNFSWDLAGGQHLNGINTLGENIADNGGLGQAYRAYQNYI..KKNNG.EE					
	* * * *	* * * *	* * * *	* * * *	* * * *	* * * *
PEX-HUM	KEKTKCMINQYSNYYWK.KAGLNVKGRKRTLGENIADNGGLREAFRAYRKWINDRRQGLEE					
	* * * *	* * * *	* * * *	* * * *	* * * *	* * * *
KELL-HU	QEAHLCLKRHYAAF..PLPSRTSFNDSLTFLENAADVGGGLAIALQAYSKRL..LRHH.GE					
	* * * *	* * * *	* * * *	* * * *	* * * *	* * * *
ECE1-HU	KRQTECMVEQYSNY..SVNG.EPVNGRHTLGENIADNGGLKAAYRAYQNWV..KKNNG.AE					
consens	KE CM QY N		NG TLGENIADNGGL	(B)	A RAY	G E

	680	690	700	710	720	730
NEP1-HU	KLLPGLDLNHNKQLFFLNFAQVWCGTYRPEYAVNSIKTDVHSPGNFRIIGTLQNSAEFSEA					
	* * * *	* * * *	* * * *	* * * *	* * * *	* * * *
PEX-HUM	PLLPGITFTNNQLFFLSYAHVRCNSYRPEAAREQVQIGAHSPPOFRVNGAISNFEEFQKA					
	* * * *	* * * *	* * * *	* * * *	* * * *	* * * *
KELL-HU	TVLPSLDLSPQQIFFRSYAQVMCRKPSPODSH...DTHSPPHLRVHGPLSSTPAFARY					
	* * * *	* * * *	* * * *	* * * *	* * * *	* * * *
ECE1-HU	HSLPTLGLTNNQLFFLGFAQVWC SVRTPESSHEGLITDPHSPSRFRVIGSLSNSKEFSEH					
consens	LP L L QLFFL	AQV C	PE	D HSP	FRV G LSN	EF

	740	750
NEP1-HU	FHCRKNSYMNPEKK.CR VW	
	* * * *	* * * *
PEX-HUM	FNCPPNSTMNRGMDSCRLW	
	* * * *	* * * *
KELL-HU	FRCARGALLNPSSR.CQLW	
	* * * *	* * * *
ECE1-HU	FRCPPGSPMNPCHK.CEVW	
consens	F C S MNP	C W

PRIMER	SEQUENCE
(1A)	5'-TGGATGGAT/CGA/CIGG/AIACIA/CA-3'
(1B)	5'-TGGATGGAT/CGA/CIGG/AIACIA/CG-3'
(2A)	5'-A/GTIGTITTT/CCCICGIGGIA/GT/AIC/TTA/TCA-3'
(2B)	5'-A/GTIGTITTT/CCCICGIGGIA/GT/AIC/TTG/CCA-3'
(3)	5'-AIIICCICCA/TC/TA/GTCIGCIG/AC/TA/GTT/CTC-3'
(4)	5'-GAT/CAAT/CT/CTIGAT/CGAA/GT/CTIAAT/CTGGATGG-3'
(5)	5'-T/CT/CACCAIATICT/GA/GCATCG/TT/CTTCATIGGG/ATG-3'

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1711 pro ala gly ile leu gln pro pro phe phe ser lys asp gln pro gln ser leu asn phe gly gly phe gly ile gly met val ile gly his glu
 CCA GCA GGG ATT CTC CAG CCG CCC TTC TTC AGC AAG GAC CAA CCA CAG TCC TTG AAT TTT GGG GGC ATC GGC ATG GTG ATT GGG CAC GAG
 600
 1801 ile thr his gly phe asp asp asn gly arg asn phe asp lys asn gly asn met leu asp trp trp ser asn phe ser ala arg his phe
 ATC ACA CAC GGC TTT GAT GAT AAT GGT CGT AAC TTT GAC AAG AAC GGC AAC ATG CTG GAC TGG TGG AGT AAC TTC TCG GCC CGG CAC TTC
 630
 1891 gln gln gln ser gln cys met ile tyr gln tyr gly asn phe ser trp gln leu ala asp asn gln asn val asn gly phe ser chr leu
 CAA CAG CAG TCG CAA TGC ATG ATC TAT CAG TAC GGC AAC TTC TCT TGG GAA CTA GCA GAC AAC CAG AAT GTG AAC GGA TTC AGT ACC CTC
 660
 1981 gly glu asn ile ala asp asn gly gly val arg gln ala tyr lys ala tyr leu arg trp leu ala asp gly gly lys asp gln arg leu
 GGG GAG AAC ATT GCC GAC AAC AAC GGA GGT GTC CGA CAG GCA TAC AAG GCT TAC CTA CCG TGG CTG GCT GAT GGC AAA GAT CAG CGA
 690
 2071 pro gly leu asn leu thr tyr ala gln leu phe phe ile asn tyr ala gln val trp cys gly ser tyr arg pro glu phe ala val gln
 CCG GGA CTC AAC ACC TAT GCC CAG CTT TTC TTC ATC AAC TAT GCC CAG GTG TGG TGT GGG TCC TAT AGG CCG GAG TTC GCC GTC CAG
 720
 2161 ser ile lys thr asp val his ser pro leu lys tyr arg val leu gly ser leu gln asn leu pro gly phe ser glu ala phe his cys
 TCC ATC AAG ACG GAC GTC CAC AGT CCT CTT AAG TAC AGG GTG CTG GGC TCA CAG AAC CTG CCA GCC TTC TCT GAG GCA TTC CAC TGC
 750
 2251 pro arg gly ser pro met his pro met lys arg cys arg ile trp ***
 CCA CGA GGC AGC CCC ATG CAC CCC ATG AAG CGA TGT CGC ATC TGG TAG CCAAGGCTGAGCTATGCTGGCCACGCCCCCGCCACCCCGGATGAGTGGTGC
 2354 GTGTAGCTGGCAGAGATGTCAGGTCCTTTGGCTGAAGGCCACCGGAGCCACCCAGCCCTCCGGCCACAGCTAGAGTGTAGCCACCCGCCACACCCGGGATGAGTGGTGC
 2473 CTGGCCCCCTCAGGCCAGTGTGGTTCAGCCAGCCAGGAGCAGTCAAGCTGCTCCATAGTGTGGCTAATATGTTCTCGAGCTTCAGACTTCAGCTAAGTAAACGC
 2925 TTC



 (cont'd)

Sequence of NL-2 cDNA from humans

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D      met val glu ser ala gly arg ala gly gln lys arg pro gly phe leu glu gly gly leu leu leu leu leu leu leu val thr
1     CTG GGG ATC GTG GAG AGC GCC CGT GCA GGG CAG AAG CGC CCG GGG TTC CTG GAG GGG GGG CTG CTG CTG CTG CTG CTG CTG CTG ACC
28
    ala ala leu val ala leu gly val leu tyr ala asp arg arg gly lys gln leu pro arg leu ala ser arg leu cys phe leu gln glu
91    GCT GCC CTG GTG GCC TTG GGT GTC CTC TAC GCC GAC CGC AGA GGG AAG CAG CTG CCA CGC CTT GCT AGC CGG CTG TGC TTC TTA CAG GAG
58
    glu arg thr phe val lys arg lys pro arg gly ile pro glu ala gln glu val ser glu val cys thr thr pro gly cys val ile ala
181   GAG AGG ACC TTT GTA AAA CGA AAA CCC CGA GGG ATC CCA GAG GCC CAA GAG GTC TGC ACC ACC CCT GGC TGC GTG ATA GCA
118
    ala ala arg ile leu gln asn met asp pro thr thr glu pro cys asp phe tyr gln phe ala cys gly gly trp leu arg arg his
271   GCC GCC AGG ATC CTC CAG AAC ATG GAC CCG ACC CCG GAA CCG TGT GAC TTC TAC CAG TTT GCA TGC GGA GGC TGG CTG CGG CGC CAC
148
    val ile pro glu thr asn ser arg tyr ser ile phe asp val leu arg asp glu leu glu val ile leu lys ala val leu glu asn ser
361   GTG ATC CCT GAG ACC AAC TCA AGA TAC AGC ATC TTT GAC GTC CTC CGC GAC GAG CTG GAG GTC AAA GCG GTG CTG GAG AAT TCG
178
    thr ala lys asp arg pro ala val glu lys ala arg thr leu tyr arg ser cys met asn gln ser val ile glu lys arg gly ser gln
451   ACT GCC AAG GAC CCG GCT GTG GAG AAG GCC AGG ACC CTG TAC CGC TCC TGC ATG AAC CAG AGT GTG ATA GAG AAG CGA CGC TCT CAG
208
    pro leu leu asp ile leu glu val val gly gly trp pro val ala met asp arg trp asn glu thr val gly leu trp glu leu glu
541   CCC CTG CTG GAC ATC TTG GAG GTG GTG GGA GGC TGG CCG GTG GCG ATG GAC AGG TGG AAC GAG ACC GTA GGA CTC GAG TGG GAG CTG
238
    arg gln leu ala leu met asn ser gln phe asn arg val leu ile asp leu phe ile trp asn asp asp gln asn ser ser arg his
631   CGG CAG CTG GCG ATG AAT AAT TCA CAG TTC AAC AGG CGC GTC ATC GAC CTC TTC ATC TGG AAC GAC GAC CAG AAC TCC AGC CGG CAC
268
    ile ile tyr ile asp gln pro thr leu gly met pro ser arg glu tyr phe asn gly ser asn arg lys val arg glu ala tyr
721   ATC ATC TAC ATA GAC CAG CCC ACC TTG GGC ATG ATG CCC TCC CGA GAG TAC TTC AAC GGC GCC AGC AAC CGG AAG GTG CGG GAA GCC
298
    leu gln phe met val ser val ala thr leu leu arg glu asp ala asn leu pro arg asp ser cys leu val gln glu asp met val gln
811   CTG CAG TTC ATG GTG TCA GTG GCC ACC TTG CTG CCG GAG GAT GCA AAC CTG CCC AGG GAC ACC TGC CTG GTG CAG GAG GAC ATG GTG CAG
328

```

FIG. 4

901 val leu glu leu glu thr gln thr ala lys ala thr val pro gln glu arg his asp val ile ala leu tyr his arg met gly leu
 CTG GAG CTG GAG ACA CAG CAG CTG GCC AAG GCC ACG ACG GTA CCC CAG GAG GAG AGA CAC GAC GAC GTC ATC ATC GCC TTG TAC CAC CGG ATG GGA
 358
 991 glu glu leu gln ser gln phe phe gly phe asn trp thr leu phe ile gln thr val ser ser val lys ile lys leu leu
 GAG CTG CAA AGC CAG TTT GGC CTG AAG GGA TTT AAC TGG ACT CTG TTC ATA CAA ACT GTG CTA TCC TCT GTC AAA ATC AAG CTG
 388
 1081 pro asp glu glu val val tyr gly ile pro tyr leu gln asn leu glu asn ile ile asp thr tyr ser ala arg thr ile gln asn
 CCA GAT GAG GAA GTG GTC TAT GGC ATC CCC TAC CTG CAG AAC CTT GAA AAC ATC ATC GAC ACC ACC GGC ACC ACC ATA CAG
 418
 1171 tyr leu val trp arg leu val val arg trp arg glu cys val gly ser leu ser gln arg phe lys asp thr arg val asn tyr arg lys ala leu phe
 TAC CTG GTC TGG CGC CTG GTG CTG GAC CGC ATT GGT AGC CTA AGC CAG AGA TTC AAG GAC ACA CGA GTG AAC TAC CGC AAG GCG CTG TTT
 448
 1261 gly thr met val glu glu val arg trp arg glu cys val gly trp val asn ser asn met glu asn ala val gly ser leu tyr val arg
 GGC ACA ATG GTG GAG GAG GTG CGC TGG CGT GAA TGT GTG GGC TAC GTC AAC AGC AAC ATG GAG AAC GCC GTG GGC TCC CTC TAC GTC
 478
 1351 glu ala phe pro gly asp ser lys ser met val arg glu leu ile asp lys val arg thr val phe val glu thr leu asp glu leu gly
 GAG CCG TTC CCT GGA GAC AGC AAG AGC ATG GTC AGA GAA CTC ATT GAC AAG GTG CCG ACA GTG TTT GTG GAG ACG CTG GAC GAG CTG GGC
 508
 1441 trp met asp glu glu ser lys lys ala gln glu lys ala met ser ile arg glu gln ile gly his pro asp tyr ile leu glu glu
 TGG ATG GAC GAG GAG TCC AAG AAG AAG GCC CAG GAG AAG GCC ATG AGC ATC CGG GAG CAG ATC GGG CAC CCT GAC TAC ATC CTG GAG
 538
 1531 met asn arg arg leu asp glu glu tyr ser asp leu asn phe ser glu asp leu tyr phe glu asn ser leu gln asn leu lys val gly
 ATG AAC AGG CGC CTG GAC GAG GAG TAC TCC AAT CTG AAC TTC TCA GAG GAC CTG TAC TTT GAG AAC AGT CTG CAG AAC CTC AAG GTG
 568
 1621 ala gln arg ser leu arg lys leu arg glu lys val asp pro asn leu trp ile ile gly ala ala val val asn ala phe tyr ser pro
 GCC CAG CCG AGC CTC AGG AAG CTT CGG GAA AAG GTG GAC CCA AAT CTC TGG ATC ATC GGG CCG GCG GTG GTC AAT GCG TTC TAC TCC
 598
 1711 asn arg asn gln ile val phe pro ala gly ile leu gln pro phe ser lys glu gln pro gln ala leu asn phe gly gly ile
 AAC CGA AAC CAG ATT GTA TTC CCT GCC GGG ATC CTC CAG CCC TTC AGC AAG GAG CAG CCA CAG GCC TTG AAC TTT GGA GGC ATT
 628

FIG. 4 (cont'd)

```

gly met val ile gly his glu ile thr his gly phe asp asp asn gly arg asn phe asp lys asn gly asn met met asp trp ser
1801 GGG ATG GTG ATC GGG CAC GAG ATC ACG CAC GAG CAC GGC TTT GAC GAC AAT GGC CGG AAC TTC GAC AAT GGC AAT ATG ATG GAT TGG TGG AGT
asn phe ser thr gln his phe arg glu gln ser glu cys met ile tyr gln tyr gly asn tyr ser trp asp leu ala asp glu gln asn
1891 AAC TTC ACC CAG CAC TTC CGG GAG CAG TCA GAG TGC ATG ATC TAC CAG TCC TGG GAC CTG GCA GAC GAA CAG AAC
val asn gly phe asn thr leu gly glu asn ile ala asp asn gly gly val arg gln ala tyr lys leu lys trp met ala glu
1981 GTG AAC GGA TTC AAC ACC CTT GGG GAA AAC ATT GCT GAC AAC GGA GGG GTG CGG CAA GGC TAC CTC AAG TGG ATG GCA GAG
gly gly lys asp gln gln thr his glu pro gly leu asp leu thr his glu gln leu phe phe ile asn tyr ala gln val trp cys gly ser tyr
2071 GGT GGC AAG GAC CAG CAG CTG CCC GGC CTG GAT CTC ACC CAT GAG CAG CTC TTC TTC ATC AAC TAT GCC CAG GTG TGG TGC GGG TCC TAC
arg pro glu phe ala ile gln ser ile lys thr asp val his ser pro lys thr arg val leu gly ser leu gln asn leu ala ala
2161 CGG CCC GAG TTC GCC ATC CAA TCC ATC AAG ACA GAC GTC CAC AGT CCC CTG AAG TAC AGG GTA CTG GGG TCG CTG CAG AAC CTG GCC GCC
phe ala asp thr phe his cys ala arg gly thr pro met his pro lys glu arg cys arg val trp ter
2251 TTC GCA GAC ACG TTC CAC TGT GCC CGG GGC ACC CCC ATG CAC CCC AAG GAG CGA TGC CGC GTG TGG TAG CCA AGG CCC TGC CGC GCT GTG
2341 CGG CCC ACG CCC ACC CGC TGC TCG GAG CCA TCT GTG CGA AGG TGC AGC TAG CGG CGA CCC AGT GTA CGT CCC GCC CCG GCC AAC CAT GCC
2431 AAG CCT GCC TGC CAG GCC TCT GCG CCT GGC CTA GGG TGC AGC CAC CTG CCT GAC ACC CAG GGA TGA GCA GTG TCC AGT GCA GTA CCT GGA
2521 CCG GAG CCC CCT TCA CAG ACA CCC GCG GGC AGT GCC CCC GTC ACA ACT CTG TAG AGA CAA TCA ACT GTG TCC TGC CCA CCC TTC AAG
2611 GTG CAT TGT CTT CCA GTA TCT ACA GCT TCA GAA CTT GAG CTA AGT AAA TGC TTT CAA AGA AAA AAA

```

FIG. 4 (cont'd)

Sequence of NL-3 cDNA from human

```

1 GGC GCT GGG AGA CAC CGG ACG CCC GCT CGG CTG CGG TGC GGC TCA GGC CCC CGC TCG GGC TCA CCG CCG GCT CGG GCG GCG
91 CGC ACC TGC CGG CTG CGG CCC CAG GGC CAT GCG GAG GCC CAC GAG GAG GCC GGC CAC GGC CAT CCC GTA GCC CAG GTG GCC CAG GTC
181 TGC ACC GCG GCG GCC TCG GCG CCG ATG GAG CCC CGG TAT TCG CTG ACG GCG CAC TAC GAT GAG TTC CAA GAG GTC AAG TAC GTG AGC CGC
      met glu pro pro tyr ser leu thr ala his tyr asp glu phe gln glu val lys tyr val ser arg
271 GGC GCG GCG GGC GGC GCG GCG GCG GCG GCG GCG GCG GCG GCG GCG GCG GCG GCG GCG GCG GCG GCG GCG GCG GCG GCG GCG
361 CCG CGC TGG AAC CGG CGC GAG GTG TGC CTG CTG TCG GGG CTG GTG TTC GGC GCG GCG GCG GCG GCG GCG GCG GCG GCG GCG GCG
      pro arg trp asn arg glu val cys leu ser gly leu val phe ala ala gly leu cys ala ile leu ala ala met leu ala leu
451 AAG TAC CTG GCG CCG GTC CCG GGC GCG GCG GCG GCG GCG GCG GCG GCG GCG GCG GCG GCG GCG GCG GCG GCG GCG GCG GCG GCG
      lys tyr leu gly pro val ala ala gly gly pro glu cys pro glu arg lys ala phe ala arg ala ala arg phe leu
541 GCC AAC CTG GAC GCC AGC ATC GAC CCA TGC CAG GAC TTC TAC TCG TTC GCC TGC GGC GGT TGG CTG CCG CAC GCC ATC CCC GAC
      ala ala asn leu asp ala ser ile asp pro cys gln asp phe tyr ser phe ala cys gly gly trp leu arg arg his ala ile pro asp
631 GAC AAG CTC ACC TAT GGC ACC ATC GCG GCA ATC GGC GAG CRA AAC GAG GAG GAG CCG CTA CCG CGC CGG CCG CCG CCG GGT GGT GGT
      asp lys leu thr tyr gly thr ile ala ala ile gly glu gln asn glu arg leu leu ala arg pro gly gly gly pro
721 GGC GCG GCC GCG CAG CGC AAG GTG CGC GCG GCG GCG GCG GCG GCG GCG GCG GCG GCG GCG GCG GCG GCG GCG GCG GCG GCG GCG
      gly gly ala ala gln arg lys val arg ala phe phe arg ser cys leu asp met arg glu ile glu arg leu gly pro arg pro met leu
811 GAG GTC ATC GAG GAC TGC GGG GCG TGG GAC CTG GCG GCG GCG GCG GCG GCG GCG GCG GCG GCG GCG GCG GCG GCG GCG GCG GCG
      glu val ile glu asp cys gly gly trp asp leu gly ala glu glu arg pro gly val ala ala arg trp asp leu asn arg leu leu
901 TAC AAG GCG CAG GGC GTG TAC AGC GCC GCC GCC GCC GCC GCC GCC GCC GCC GCC GCC GCC GCC GCC GCC GCC GCC GCC GCC
      tyr lys ala gln gly val tyr ser ala ala leu phe ser leu thr val ser leu asp asp arg asn ser ser arg tyr val ile arg
991 ATT GAC CAG GAT GGG CTC ACC CTG CCA GAG AGG ACC CTG TAC CTC GCT CAG GAT GAG GAC AGT GAG AAG GTC CTG GCA GCA TAC AGG GTG
      ile asp gln asp gly leu thr leu tyr leu ala gln asp glu ser glu lys val leu ala ala tyr arg val
1081 TTC ATG GAG CGA GTG CTC AGC CTC GGT GCA GAC GCT GTG GAA CAG AAG GCC CAA GAG ATC CTG CAA GTG GAG CAG CAG CTG GCC AAC
      phe met glu arg val leu ser leu leu gly ala asp ala val glu gln lys ala gln glu ile leu gln val glu gln leu ala asn

```

FIS - 5

ile thr val ser glu tyr asp asp leu arg arg asp val ser ser met tyr asn lys val thr leu gly gln leu gln lys ile thr pro
 1171 ATC ACT GTG TCA GAG TAT GAC GAC GAC CTA CGG CGA GAT GTC AGC TCC ATG TAC AAC AAG GTG ACG CTG GGG CAG CTG CAG AAG ATC ACC CCC
 382
 his leu arg trp lys trp leu leu asp gln ile phe gln glu asp phe ser glu glu glu val val leu leu ala thr asp tyr met
 1261 CAC TTG CGG TGG AAG TGG CTG CTA GAC CAG ATC TTC CAG GAG GAC TTC TCA GAG GAA GAG GTG GTG CTG GCG ACA GAC TAC ATG
 412
 gln gln val ser gln leu ile arg ser thr pro his arg val leu val his asn tyr leu val trp arg val val val leu ser glu his
 1351 CAG CAG GTG TCG CAG CTC ATC CGC TCC ACA CCC CAC CGG GTC CTG CAC AAC TAC CTG GTG TGG CGC GTG GTG GTC CTG AGT GRA CAC
 442
 leu ser pro pro phe arg glu ala leu his glu leu ala gln glu met glu gly ser asp lys pro gln glu leu ala arg val cys leu
 1441 CTG TCC CCG CCA TTC CGT GAG GCA CTG CAG GAG CTG GCA CAG GAG ATG GAG GGC AGC GAC AAG CCA CAG GAG CTG GCC GTC TGC TTG
 472
 gly gln ala asn arg his phe glu met ala leu gly ala leu phe val his glu his phe ser ala ala ser lys ala lys val gln gln
 □ 1531 GGC CAG GCC AAT CGC CAC TTT GGC ATG GCG CTT GGC GGC CTC TTT GTA CAT GAG CAC TTC TCA GCT GCC AGC ARA GCC AAG GTG CAG CAG
 502
 leu val glu asp ile lys tyr ile leu gly gln arg leu glu glu leu asp trp met asp ala glu thr arg ala ala arg ala lys
 1621 CTA GTG GAA GAC ATC AAG TAC ATC CTG GGC CAG CGC CTG GAG GAG CTG GAC TGG ATG GAC GCC GAG ACC AGC GCT GCT CGG GCC AAG
 532
 leu gln tyr met met val met val gly tyr pro asp phe leu leu lys pro asp ala val asp lys glu tyr glu phe glu val his glu
 1711 CTC CAG TAC ATG ATG GTG ATG GTC GGC TAC CCG GAC TTC CTG CTG AAA CCC GAT GCT GTG GAC AAG GAG TAT GAG TTT GAG GTC CAT GAG
 562
 lys thr tyr phe lys asn ile leu asn ser ile arg phe ser ile gln leu ser val lys lys ile arg gln glu val asp lys ser thr
 1801 AAG ACC TAC TTC AAG AAC ATC TTG AAC AGC ATC CGC TTC AGC ATC CAG CTC TCA GTT AAG AAG ATT CGG CAG GAG GTG GAC AAG TCC ACG
 592
 trp leu leu pro pro gln ala leu asn ala tyr tyr leu pro asn lys asn gln met val phe pro ala gly ile leu gln pro thr leu
 1891 TGG CTG CTC CCC CCA CAG GCG CTC AAT GCC TAC TAT CTA CCC AAC AAG AAC CAG ATG GTG TTC CCC GCG GGC ATC CTG CAG CCC ACC CTG
 622
 tyr asp pro asp phe pro gln ser leu asn tyr gly gly ile gly thr ile ile gly his glu leu thr his gly tyr asp asp trp gly
 1981 TAC GAC CCT GAC TTC CCA CAG TCT CTC AAC TAC GGC GGC ATC ACC ACC ATT GGA CAT GAG CTG ACC CAC GGC TAC GAC GAC TGG GGG
 652

FRS - 5 (cont'd)

gly gln tyr asp arg ser gly asp arg ser gly asp arg ser gly asp arg ser arg phe leu arg lys ala glu cys ile val arg
 2071 GGC CAG TAT GAC GAC CGC TCA GGC AAC GGG AAC TGG TGG ACC GAG CAC TGG TGG ACC GAG CAC TCC TAC TAC AGC CGC TTC CGA AAG GCT GAG TGC ATC GTC
 682
 leu tyr asp asn phe thr val tyr asp gln lys trp val arg glu his trp thr leu gly glu asn ile ala asp met gly gly leu lys leu
 2161 CTC TAT GAC AAC TTC ACT GTC TAC AAC TGG GTG AAC GGG AAA CAC ACC CTT GGG GAG AAC ATC GCA GAT ATG GGC GGC CTC AAG
 712
 ala tyr his ala tyr gln lys trp val arg glu his gly pro glu his pro leu lys thr thr his asp gln leu phe phe
 2251 GCC TAC CAC GCC TAT CAG AAG TGG GTG CAG CAC GGC CCA GAG CAC CCA CTT CCC CGG CTC AAG TAC ACA CAT GAC CAG CTC TTC TTC
 742
 ile ala phe ala gln asn trp cys ile lys arg arg ser gln ser ile tyr leu gln val leu thr asp lys his ala pro glu his tyr
 2341 ATT GCC TTT GCC CAG AAC TGG TGC ATC AAG CCG CCG TCG CAG TCC ATC TAC TAC CTG ACT GAC AAG CAT GCC CCT GAG CAC TAC
 772
 arg val leu gly ser val ser gln phe glu phe gly arg val leu his cys pro lys val ser pro met asn pro ala his lys cys
 2431 AGG GTG CTG GGC AGT GTG TCC CAG TTT GAG GAG TTT GGC CGG GTT TTA CAC TGT CCA AAG GTC TCA CCC ATG AAC CCT GCC CAC AAG TGT
 775
 ser val trp ter
 2521 TCC GTG TGG TGA CCC TGG CTG CCC TGC ACC GCC TGC ACC CCC CCA CTG CCC CCG CAC GAA TCA CCT GCT GGC TAC CCG GGC AGG CAT GCA CCC
 2611 GGT GCC AGC CCC GCT CTG GGC ACC ACC TGC CTT CCA GCC CCT CCA GGA CCC GGT GCT GCT GCT GCT CAC TTC AGG AGG GGC CTG GAG
 2701 CAG GGT GAG GCT GGA CTT TGG GGG GCT GTG AGG GAA ATA TAC TGG GGT CCC CAG ATT CTG CTC TRA GGG GGC CAG ACC CTC TGC CAG GCT
 2791 GGA TTG TAC GGG CCC CAC CTT CGC TGT GTT CTT GCT GCA AGT CTG GTC AAA TAA ATC ACT GCA CTG TTA AAA AAA AAA

FIG. 5 (cont'd)

Sequence comparison between NEP, NL1, NL2 and NL3

```

1           10           20           30           40
NEP-HUM MG.....KSESQMDITDINTPKPKKKQRWTPLEISLSVLVLL..LTII...AV
*
NL1-MOU MV.....ERAGWCRKKSPGFVEYGLMVLLLLLLGAIVTLG.V..FYSI.GKQL
**          * * * * * * * * * * * * * * * * * * * * * * * * * * * *
NL2-HUM MV.....ESAGRAGQKRPGFLEGGLLLLLLLLVTAALVALGVL..YADRRGKQL
*
NL3-HUM MEPPYSLTAHYDEFQEVKYVSRGAGGARGASLPPGFPLGAARSATGARSGLPRWNRREV

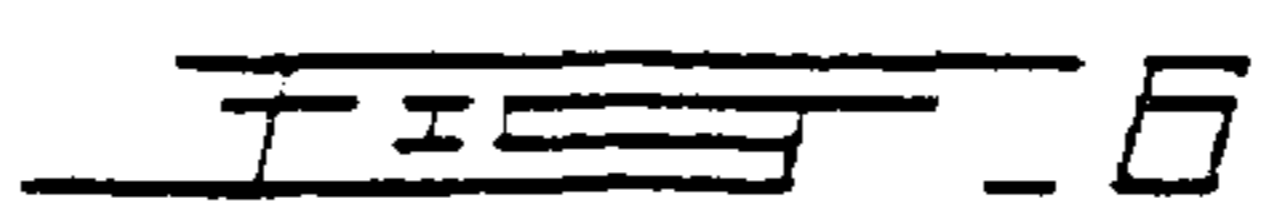
           50           60           70
NEP-HUM TMIA.....LYATYDD.....GICKSSDCIKSAARLIQ.NMDATT
*
NL1-MOU PLLTSL.....LHESWDERTVVKR...ALRDSSLKSDICTTPSCVIAAARILE.NMDQSR
* * * * * * * * * * * * * * * * * * * * * * * * * * * *
NL2-HUM PRLASR.....LCFLQEERTFVKRKPRGIPEAQEVSEVCTTPGCVIAAARILQ.NMDPTT
*
NL3-HUM CLLSGLVFAAGLCAILAAMLALKYLGPVAAGGGACPEGC PERKAFARAARFLAANLDASI

           80           90           100          110          120          130
NEP-HUM EPCTDFFKYACGGWLKRNVIPETSSRYGNFDILRDELEVVLKDVLPQEPKTEDIVAVQ.KA
** * * * * * * * * * * * * * * * * * * * * * * * * * * * *
NL1-MOU NPCENFYQYACGGWLRHHVIPETNSRYSVFDILRDELEVILKGVLEDSTSQHRPAVE.KA
** * * * * * * * * * * * * * * * * * * * * * * * * * * * *
NL2-HUM EPCDDFYQFACGGWLRRHVIPETNSRYSIFDVLRLDELEVILKAVLENSTAKDRPAVE.KA
** * * * * * * * * * * * * * * * * * * * * * * * * * * * *
NL3-HUM DPCQDFYSFACGGWLRRHAI PDDKLT YGTIAAIGE QNEERLRRL LARPGGGPGGAAQRKV

           140          150          160          170          180          190
NEP-HUM KALYRSCINESAIDSRGGEPLLKLLPDIYGWPVATENWEQKYGASWTAEKAI AQLNSKYG
* * * * * * * * * * * * * * * * * * * * * * * * * * * *
NL1-MOU KTLYRSCMNQSVIEKRDSEPLLSVLKMGVGGWPVAMDKWNETMGLKWELERQLAVLNSQFN
* * * * * * * * * * * * * * * * * * * * * * * * * * * *
NL2-HUM RTLYRSCMNQSVIEKRGSQPLLDILEVVGWPVAMDRWNETVGLEWELERQLALMNSQFN
* * * * * * * * * * * * * * * * * * * * * * * * * * * *
NL3-HUM RAFFRSCLDMREIERLGP RP MLEVI EDCGGWDLGGAERPGVAARWDLNRLLYKAQGVYS

           200          210          220          230          240          250
NEP-HUM KKVLINLFEVGTDDKNSVNHVIHIDQPRGLPSRDYECTGIYKEACTAYVDFMISVARLI
* * * * * * * * * * * * * * * * * * * * * * * * * * * *
NL1-MOU RRVLIDLFIWNDDQNSSRHVIYIDQPTLGMP SREY YFQEDNNHKVRKAYLEFMTSVATML
* * * * * * * * * * * * * * * * * * * * * * * * * * * *
NL2-HUM RRVLIDLFIWNDDQNSSRHIIYIDQPTLGMP SREY YFNGGSRKVRREAYLQFMVSVATLL
* * * * * * * * * * * * * * * * * * * * * * * * * * * *
NL3-HUM AAALFSLTVSLDDRNSRYVIRIDQDGLTLPERTLYLAQDEDESE..KVLAA YRVFMERVL

```



260 270 280 290 300 310
 NEP-HUM RQEERLPIDENQLALEMNVMELEKEIANATAKP . . EDRNDPMLLYNKMTLAQIQNNFSL
 * * * * *
 NL1-MOU RKDQNLKESAMVREEMADEVLELETHLANATVPQ . . EKRHDVTALYHFMDLMELQERFGL
 * * * * *
 NL2-HUM REDANLPRDSCLVQEDMVQVLELETQLAKATVPQ . . EERHDVIALYHRMGLEELQSQFGL
 * * * * *
 NL3-HUM SL . . . LGADAV . . EQKAQEILQVEQQLANITVSEYDDLRRDVSSMYNKVTLGQLQKITP .

320 330 340 350 360 370
 NEP-HUM EINGKPF SWLNFTNEIMSTVNI SITNEEDVVVYAPEYLTCLKPILTKYSARDLQNLMSWR
 * * * * *
 NL1-MOU KGFNWTLFIQNVLSSVEVELFPDEEVVYGI PYLENLEDIIDSYSARTMQNYLVWR
 * * * * *
 NL2-HUM KGFNWTLFIQTVLSSVKIKLLPDEEVVYGI PYLQNLNIIDTYSARTIQNYLVWR
 * * * * *
 NL3-HUM HLRWKWLLDQIF . . . QEDFSEEEVLLATDYMQQVSQLIRSTPHRVLHNYLVWR

380 390 400 410 420 430
 NEP-HUM FIMDLVSSLSRITYKESRNAFRKALYGGTSETATWRRRCANYVNGNMENAVGRLYVEAAFAG
 * * * * *
 NL1-MOU LVLDRIGSLSQRFKEARVDYRKALYGGTVEEVRWRECVSYVNSNMESAVGSLYIKRAFSK
 * * * * *
 NL2-HUM LVLDRIGSLSQRFKDTRVMYRKALFGTMVEEVRWRECVGYVNSNMENAVGSLYVREAFPG
 * * * * *
 NL3-HUM VVVVLSLSEHLSPPFREALHELAQEMEGSDKPQELARVCLGQANRHF GMALGALFVHEHFSA

440 450 460 470 480 490
 NEP-HUM ESKHVVEDLIAQIREVFIQTLDDLTWMDAETKKRAEEKALAIKERIGYPDDIVSNDNK . L
 * * * * *
 NL1-MOU DSKSTVRELIEKIRSVFVDNLDELNWMDEESKKAQEKAMNIREQIGYPDYILEDNNKHL
 * * * * *
 NL2-HUM DSKSMVRELIDKVRTVFVETLDELGWMDEESKKAQEKAMSIREQIGHDPDYILEEMNRRLL
 * * * * *
 NL3-HUM ASKAKVQQLVEDIKYILGQRLEELDWMDAETRAAARAKLQYMMVMVGYPDBFLKPD . . V

500 510 520 530 540 550
 NEP-HUM NNEYLELNYKEDEYFENIIQNLKFSQSKQLKKLREKVDKDEWISGAAVVNAFYSSGRNQI
 * * * * *
 NL1-MOU DEEYSSLTFYEDLYFENGLQNLKNAQRS LKKLREKVDQNLWIIGA AVVNAFYSPNRNQI
 * * * * *
 NL2-HUM DEEYSNLNFSLEDLYFENSLQNLKVGAQRS LRKLRKVDPNLWIIGA AVVNAFYSPNRNQI
 * * * * *
 NL3-HUM DKE . YEFVHEKTYFKNILNSIRFSIQLSVKKIRQEVKSTWLLPPQALNAYYLPNKNQM

FIG 15 - B (cont'd)

```

          560      570      580      590      600      610
NEP-HUM  VFPAGILQPPFFSAQQSNLSLNYGGIGMVGHEITHGFDDNGRNFNKGDLVDWWTQQSAS
          ***** *  *** -***** * -   ***  **
NL1-MOU  VFPAGILQPPFFSKDQPQSLNFGGIGMVGHEITHGFDDNGRNFDKNGNMLDWWSNESAR
          ***** **  *****
NL2-HUM  VFPAGILQPPFFSKEQPQALNFGGIGMVGHEITHGFDDNGRNFDKNGNMMDWWSNESTQ
          ***** *  *  *  *  *  *  *  *  *  *  *  *
NL3-HUM  VFPAGILQPTLYDPDFPQSLNFGGIGTIIGHELTHGYDDWGGQYDRSGNLLHWWTEASYS

```

```

          620      630      640      650      660      670
NEP-HUM  NFKEQSQCMVYQYGNFSWDLAGGQHLNGINTLGENIADNGGLGQAYRAYQNYIKKNGEEK
          *  ***** *  *  *  *  *  *  *  *  *  *
NL1-MOU  HFQQQSQCMIYQYGNFSWELADNQNNGFSTLGENIADHGGVVRQAYKAYLRWLADGGKDQ
          **  ** ***** *  *  *  *  *  *  *  *  *  *  *  *
NL2-HUM  HFREQSECMIYQYGNYSWDLADEQNNGENTLGENIADNGGVVRQAYKAYLKWMAEGGKDQ
          *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
NL3-HUM  RFLRKAECIVRLYDNFT...VYNQRVNGKHTLGENIADMGGKLAYHAYQKWVREHGPEH

```

```

          680      690      700      710      720      730
NEP-HUM  LLPGLDLNHKQLFFLNFAQVWCGTYRPEYAVNSIKTDVHSPGNFRIIGTLQNSAEFSEAF
          ***** *  ***** *  ***** *  ***** *  *****
NL1-MOU  RLPGLNLTYAQLFFINYAQVWCGSYRPEFAVQSIKTDVHSPLKYRVLGSLQNLPGFSEAF
          ***** **  ***** ***** ***** ***** ***** *
NL2-HUM  QLPGLDLTHEQLFFINYAQVWCGSYRPEFAIQSIKTDVHSPLKYRVLGSLQNLAAFADTF
          **  *  *  ***** *  *  *  *  *  *  *  *  *
NL3-HUM  PLPRLKYTHDQLFFIAFAQNWCIKRRSQSIYLQVLTDKHAPEHYRVLGSVVSQFEEFGRVL

```

```

          740      750
NEP-HUM  HCRKNSYMNPEKKCRVW
          **  *  *  *  *  *  *
NL1-MOU  HCPRGSPMHPMKRCRIW
          **  ** ***** *  *  *
NL2-HUM  HCARGTEMHPKERCRVW
          **  **  *  *  *  *
NL3-HUM  HCPKVSPMNPAAHKCSVW

```

FIG 16 - 6 (cont'd)

NL1 in the TESTIS

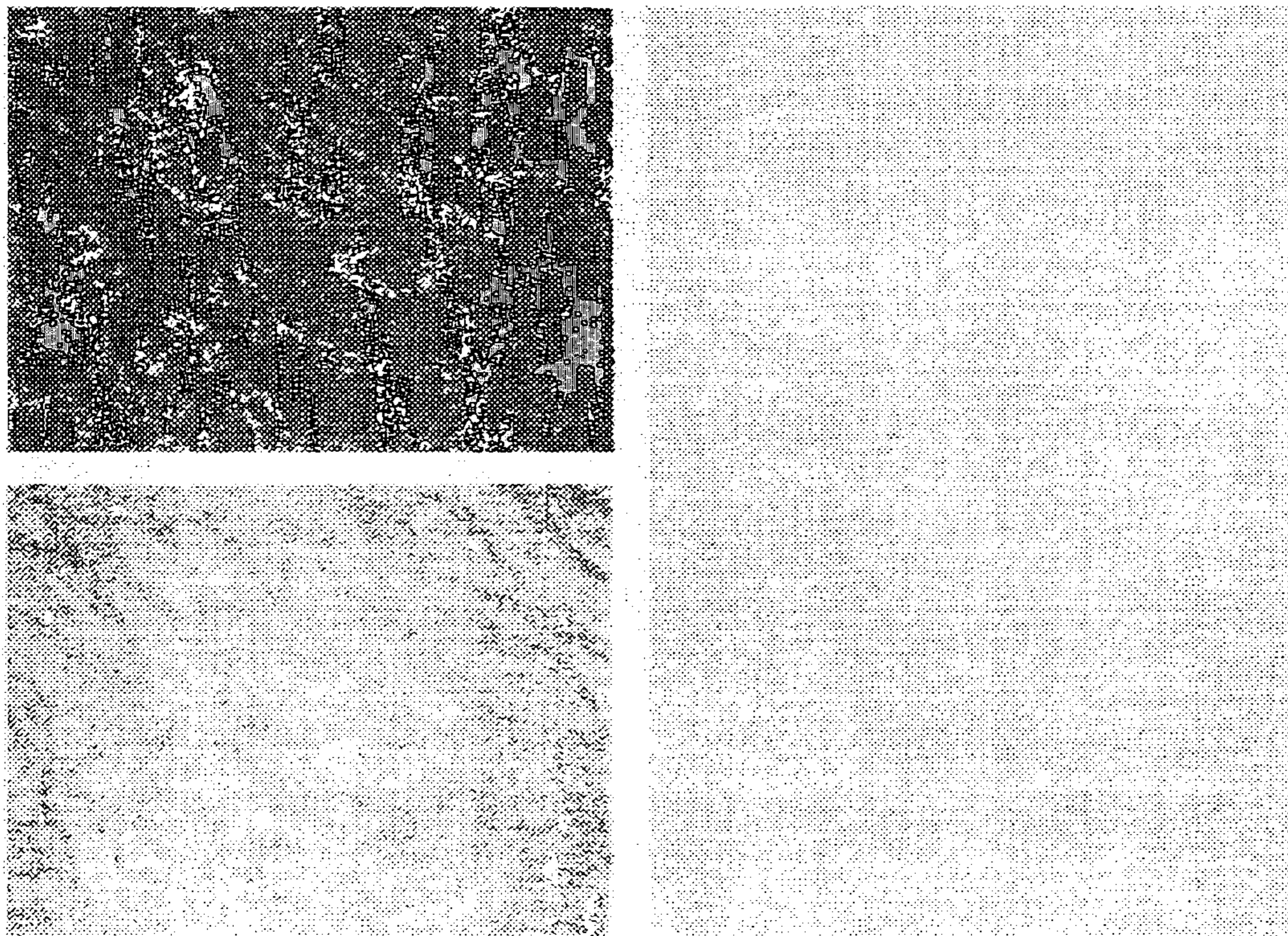


FIG. 7

Expression of PEX and NL-3 in normal and Hyp mouse embryos

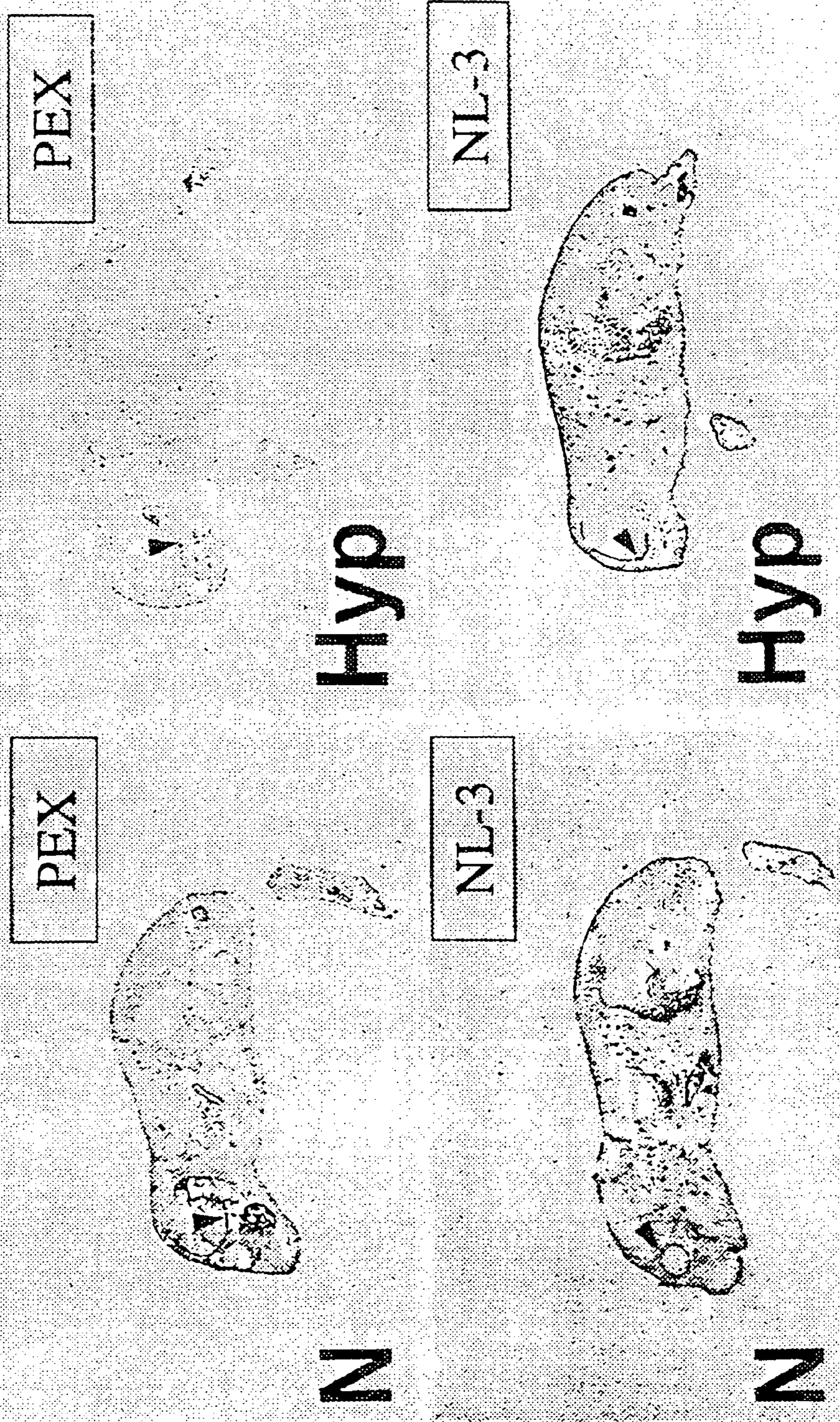


FIG. 18

NL3 in the BRAIN

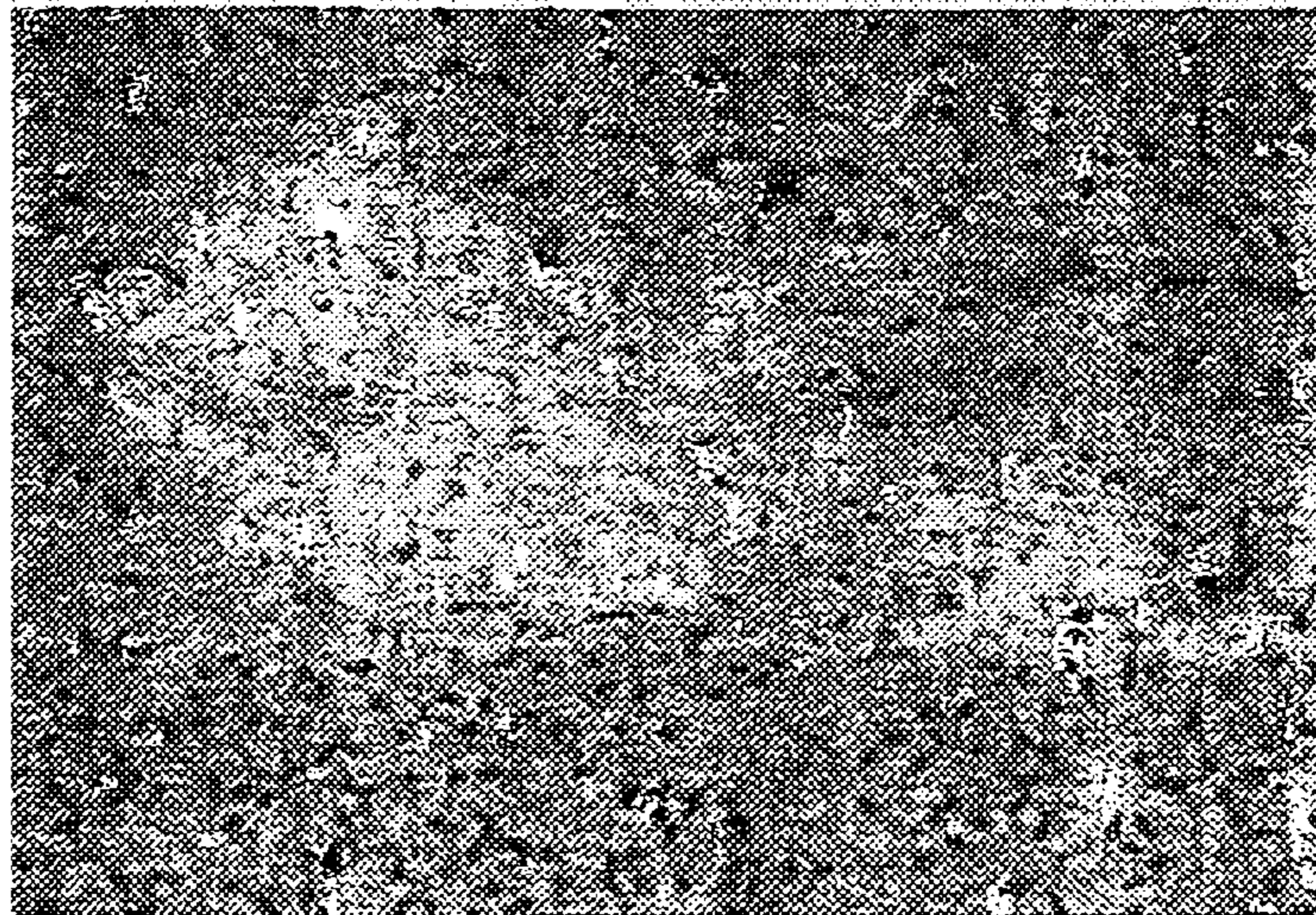
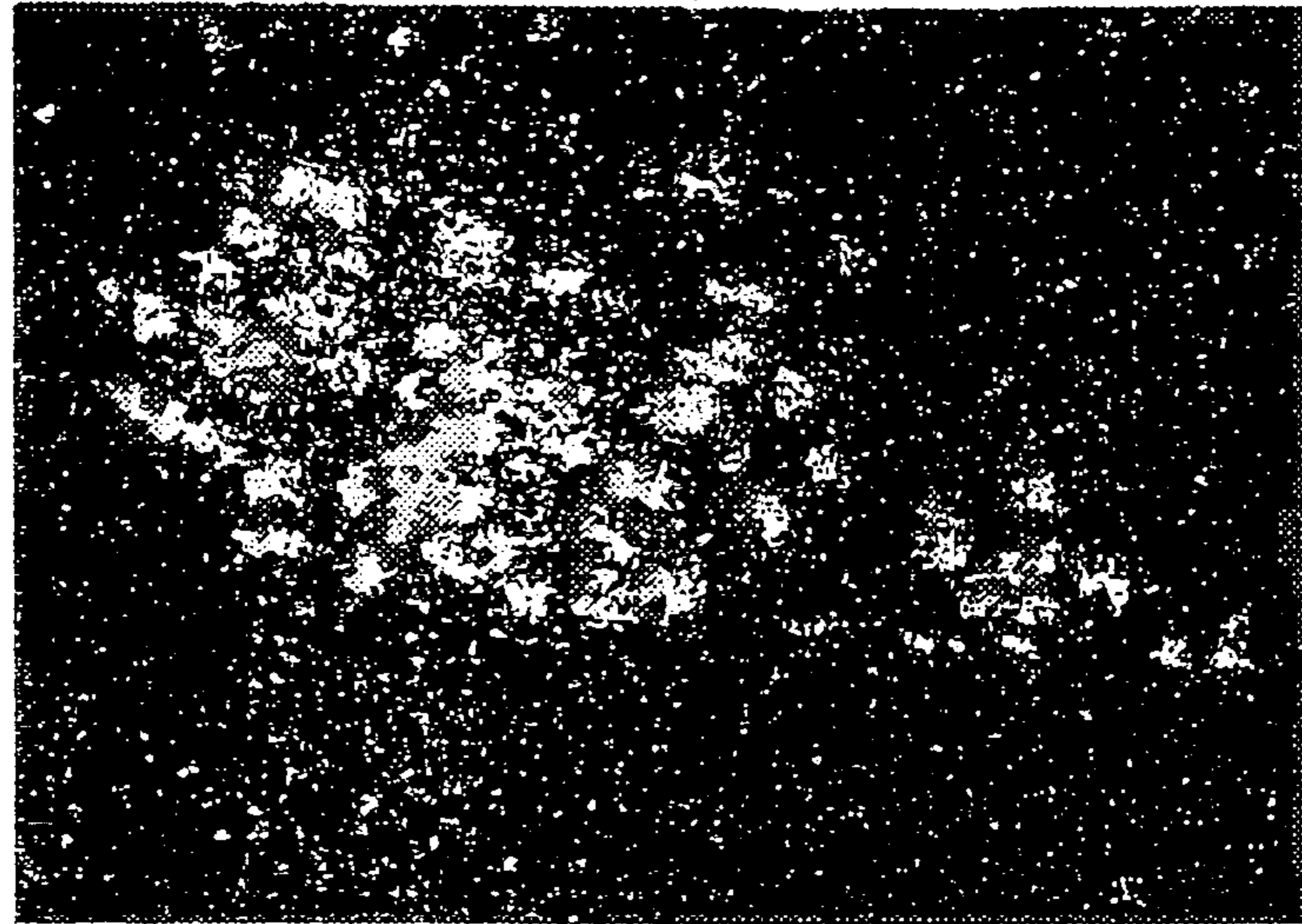


FIG. 9

Structure and expression of NL-1

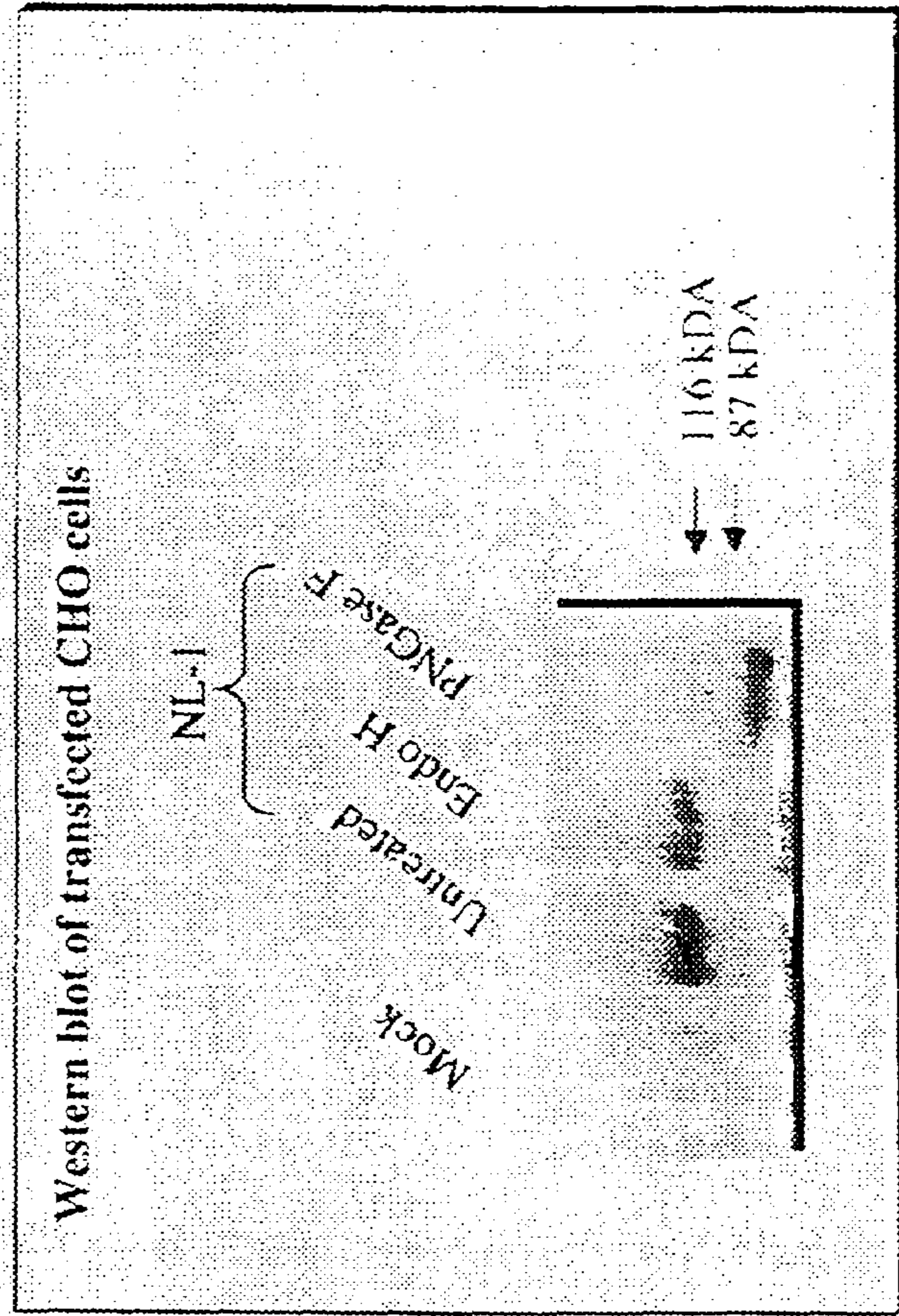
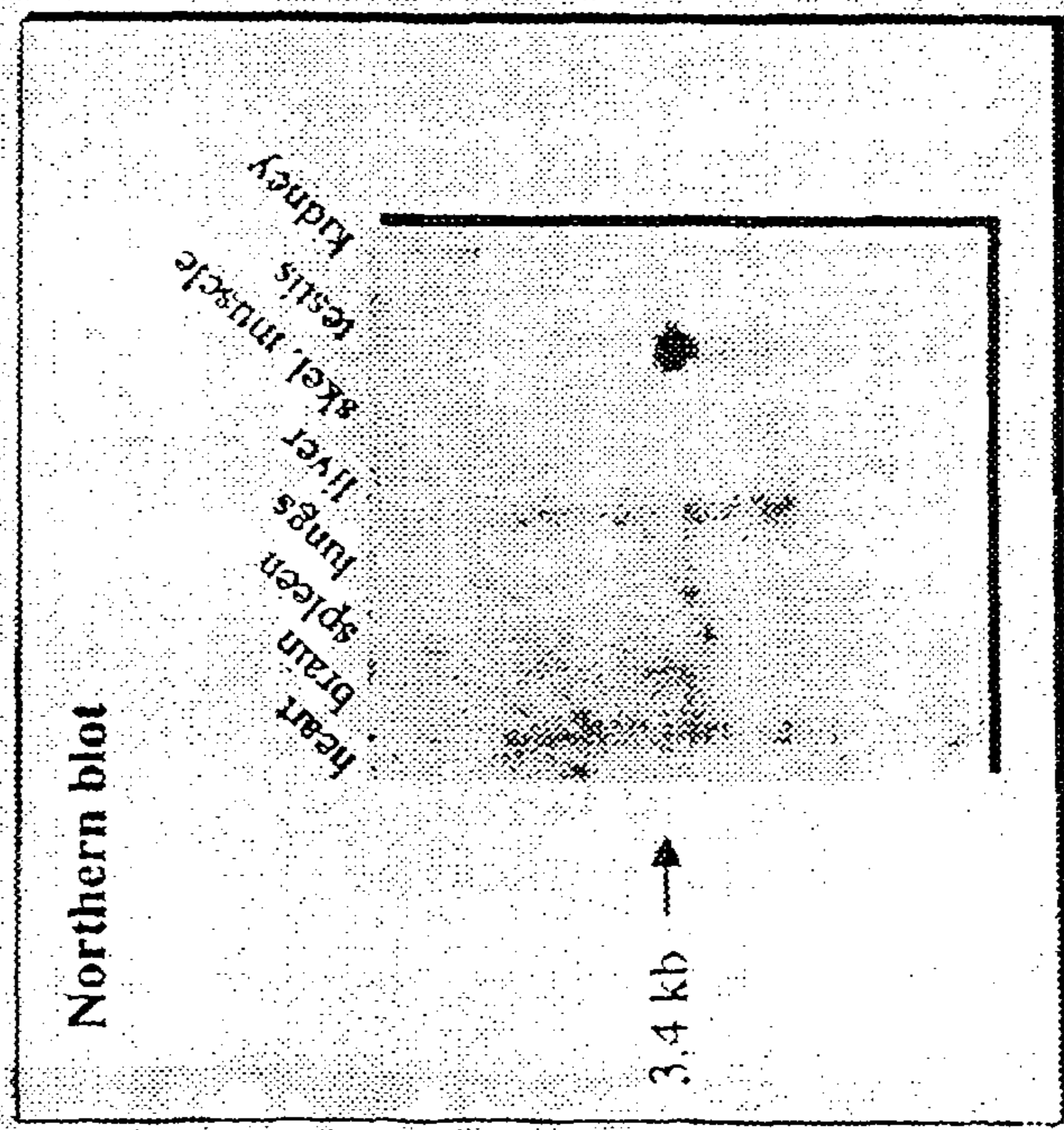
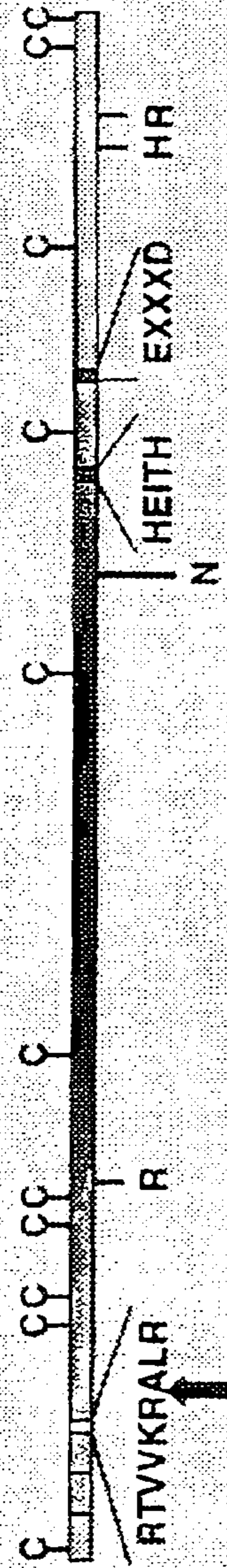


FIG. 10

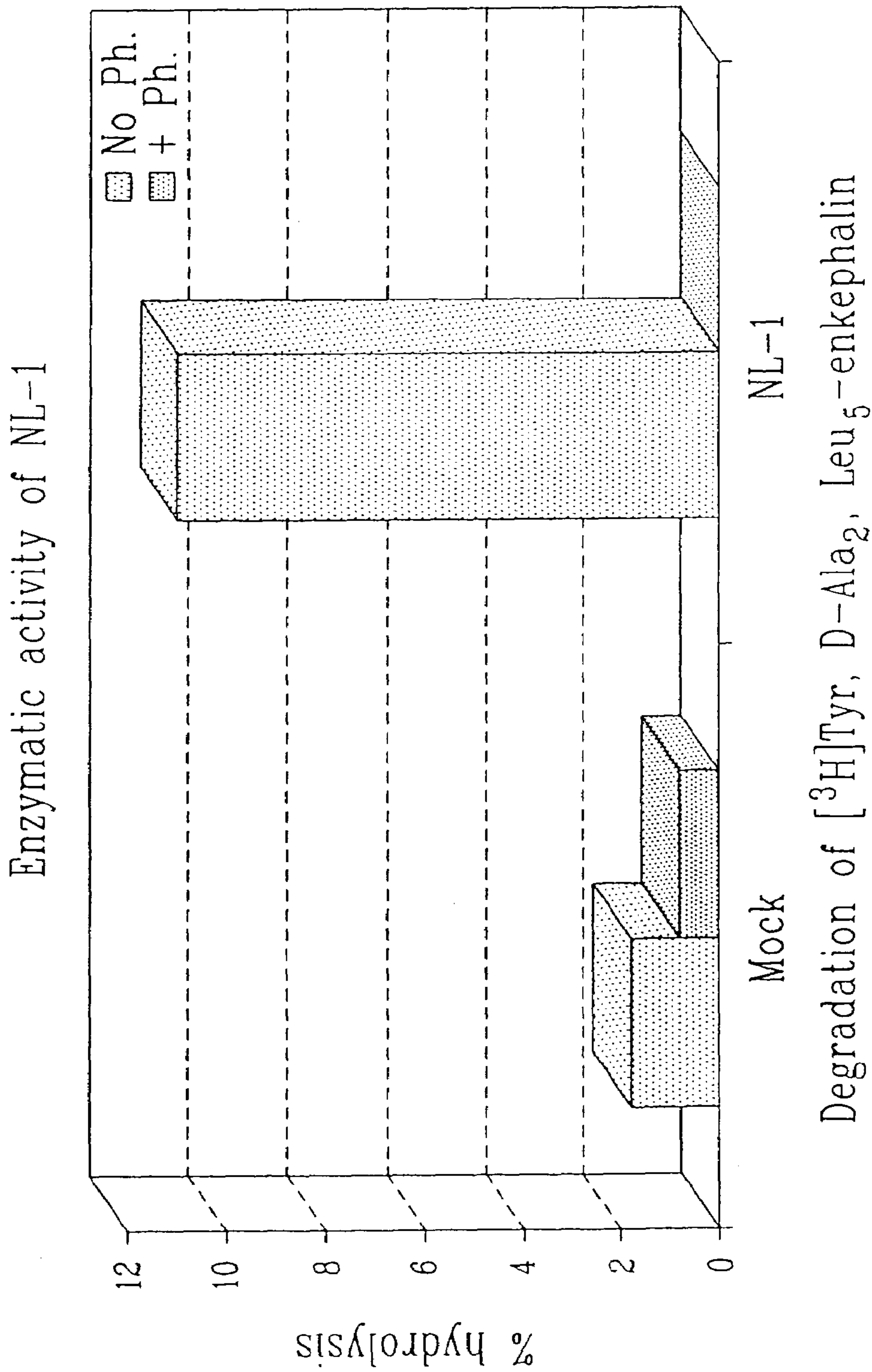


FIG. 11

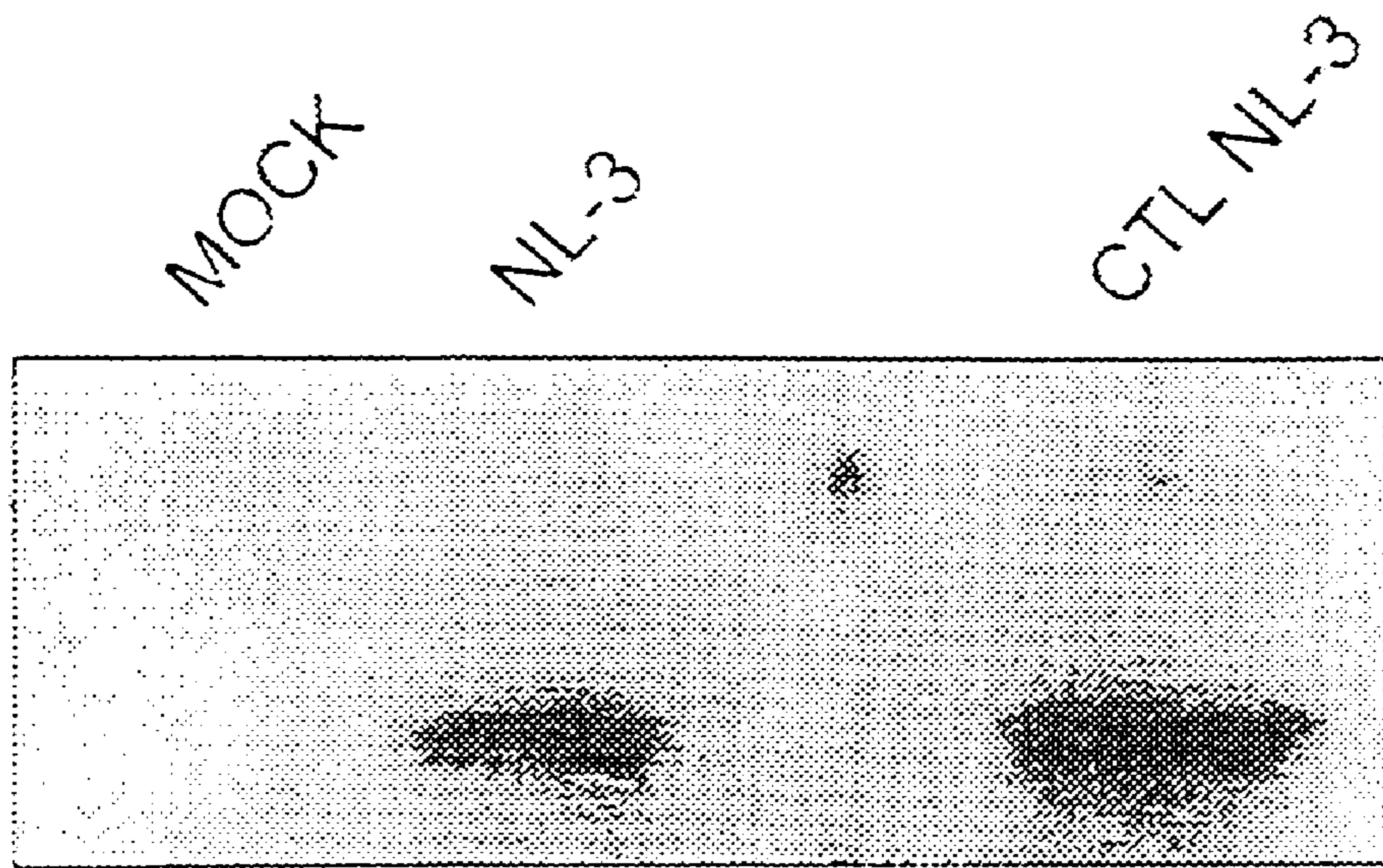


FIG. 12

METALLOPROTEASES OF THE NEPRILYSIN FAMILY

This application is the U.S. National Phase of International Application PCT/CA00/00147, filed Feb. 11, 2000, published in English under PCT Article 21(2), which designated the U.S. PCT/CA00/00147 claims priority to Canadian Patent Application No. 2,260,376, filed Feb. 11, 1999. The entire content of these applications are incorporated herein by reference.

BACKGROUND OF THE INVENTION

Peptides are used by cells from yeast to mammals to elicit physiological responses. The use of peptides as messengers usually involves the following steps: 1) production and release of the peptide by a specific cell, 2) interaction of the peptide with a receptor on the surface of the target cell, and 3) degradation of the peptide to terminate its action. The first and last steps of this scheme require the participation of proteases/peptidases. There is increasing evidence that membrane-associated zinc-metallopeptidases play important roles in both of these steps. Although activation of prohormone precursors into bioactive peptides is generally performed by proteases of the subtilisin family located in the Trans-Golgi Network or in secretory granules of the cell (for a review see: (Seidah and Chretien, 1995)) a few peptides need a final processing step. This step involves the action of membrane-associated zinc-metallopeptidases. Two cases are particularly well documented: angiotensin-converting enzyme (ACE) which cleaves inactive angiotensin I into angiotensin II (Corvol and Williams, 1997) and endothelin-converting enzymes (ECEs) which cleave isoforms of big endothelins into endothelins (Turner, 1997a). In addition to their role in peptide activation, cell surface zinc-metallopeptidases have also been implicated in the termination of the peptidergic signal by breaking down the active peptides into inactive fragments. One of the best known of these peptidases is probably Neutral Endopeptidase-24.11 (Neprilysin, NEP) that has been implicated in the physiological degradation of several bioactive peptides (Kenny, 1993). Interestingly, NEP and the ECEs show significant structural similarities and appear to be members of a family of peptidases that also includes PEX, a newly discovered and not yet characterized peptidase, and the KELL blood group protein (Turner and Tanzawa, 1997b). Because of their important role as regulators of bioactive peptide activity, these enzymes (more specifically NEP and the ECEs) have been identified as putative targets for therapeutic intervention, similar to the way ACE inhibitors are used to control blood pressure. The recent discovery of PEX, another member of the family, which appears to be involved in phosphate homeostasis, raised the possibility that other yet unknown members might exist.

Members of the NEP-like family are type II membrane proteins consisting of three distinct domains: a short NH₂-terminal cytosolic sequence, a single transmembrane region, and a large extracellular or ectodomain responsible for the catalytic activity of the enzyme. There are potential N-glycosylation sites and cysteine residues that are involved in disulfide bridges stabilizing the conformation of the active enzyme. These enzymes are metalloenzymes with a Zn atom in their active site. As such, they belong to the zincin family of peptidases which is characterized by the active site consensus sequence HEXXH (Hooper, 1994), where the two histidine residues are zinc ligands. In members of the NEP-like family of peptidases, the third zinc ligand is a

glutamic acid residue located on the carboxy-terminus side of the consensus sequence. This characteristic puts them in the gluzincin sub-family (Hooper, 1994). The model enzyme for gluzincins is thermolysin (TLN) a bacterial protease whose 3D structure has been determined by X-ray crystallography (Holmes and Matthews, 1982). The active site of NEP has been extensively studied by site-directed mutagenesis and several residues involved in zinc binding (Devault et al., 1988b; Le Moual et al., 1991; Le Moual et al., 1994), catalysis (Devault et al., 1988a; Dion et al., 1993), or substrate binding (Vijayaraghavan et al., 1990; Beaumont et al., 1991; Dion et al., 1995; Marie-Claire et al., 1997) have been identified (for a recent review see Crine et al., 1997).

SUMMARY OF THE INVENTION

Here, we developed an RT-PCR strategy to look for other members of this important family of peptidases. This strategy allowed the molecular cloning and characterization of three additional NEP-like (NL) metallopeptidases (called NL-1, NL-2 and NL-3). Knowledge obtained through these studies allows the generation of reagents (nucleic acid probes and primers, antibodies and active recombinant enzymes) for further biochemical characterization of these enzymes and their pattern of expression and will greatly help the rational design of specific inhibitors that could be used as therapeutic agents.

Accordingly, the present invention relates to the following products:

- A. Degenerate primers for screening new NEP-related enzymes;
- B. NL-1, NL-2 and NL-3 proteins as NEP-related enzymes;
- C. Nucleic acids encoding these enzymes;
- D. Antibodies directed against the enzymes;
- E. Recombinant vectors comprising the nucleic acids encoding the enzymes and hosts transformed therewith;
- F. Fragments of the nucleic acids useful as probes or primers to hybridize and detect the presence of an NL-1, NL-2 and NL-3 genes, or to hybridize and amplify and produce gene fragments;
- G. Soluble forms of NL-1, NL-2 and NL-3; and
- H. Nucleic acids comprising the N-terminal part of NL-1 or NL-2 which terminates with a sequence encoding a furin recognition site, such nucleic acids being useful for making a fusion protein with the ectodomain of any protein of interest, and for releasing a soluble form of that protein of interest (containing the ectodomain) in the medium.

Also the present invention relates to the following methods:

- A. A method for screening NEP-related enzymes that make use of degenerate primers or probes selected from a region of NEP family members in a highly conserved region, namely around the zinc-binding sites; and
- B. A method for producing NL-1, NL-2 or NL-3 that includes the steps of culturing the above recombinant host and recovering NL-1, NL-2 and NL-3 gene products therefrom.

The present invention will be described hereinbelow by referring to specific embodiments and appended figures, which purpose is to illustrate the invention rather than to limit its scope.

In the first section, general procedures leading to the identification and localization of NL-1, NL-2 and NL-3 are given. In the second section, slightly different procedures are given for completing or reiterating the work performed on NL-1.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1: Amino acid sequence comparison of human NEP (SEQ ID NO: 1), PEX (SEQ ID NO: 2), KELL (SEQ ID NO: 3) and ECE1 (SEQ ID NO: 4) peptidases. Amino acid sequences in boxes are those used to design the oligonucleotide primers. Numbers and arrows under the sequences identify the primer and its orientation.

FIG. 2: Sequences of the oligonucleotide primers used in the PCR reactions (1A: SEQ ID NO: 5; 1B: SEQ ID NO: 6; 2A: SEQ ID NO: 7; 2B: SEQ ID NO: 8; 3: SEQ ID NO: 9; 4: SEQ ID NO: 10; and 5: SEQ ID NO: 11).

FIG. 3: Nucleotide (SEQ ID NO: 12) and amino acid (SEQ ID NO: 13) sequence of the mouse NL-1 cDNA. The sequence of the DNA fragment obtained by PCR is in brackets.

FIG. 4: Partial nucleotide (SEQ ID NO: 14) and amino acid sequence (SEQ ID NO: 15) of the human NL-2 cDNA. The sequence of the DNA fragment obtained by PCR is in brackets.

FIG. 5: Partial nucleotide (SEQ ID NO: 16) and amino acid sequence (SEQ ID NO: 17) of the human NL-3 cDNA.

FIG. 6: Amino acid sequence comparison of NEP (SEQ ID NO: 1), NL-1 (SEQ ID NO: 13), NL-2 (SEQ ID NO: 15) and NL-3 (SEQ ID NO: 17) peptidases.

FIG. 7: In situ hybridization of mouse testis sections using NL-1 as a probe.

FIG. 8: In situ hybridization of mouse sections using mouse NL-3 as a probe.

FIG. 9: In situ hybridization of mouse spinal chord sections

FIG. 10: Expression of NL-1 in mammalian cells.

FIG. 11: Activity of recombinant soluble NL-1.

FIG. 12: Expression of a soluble form of NL-3 using NL-1 amino-terminal domain.

DESCRIPTION OF THE PREFERRED EMBODIMENTS OF THE INVENTION

Section 1)

Materials and Methods

DNA and RNA manipulations

All DNA manipulations and Northern blot analysis were performed according to standard protocols (Ausubel et al., 1988; Sambrook et al., 1989).

mRNA purification and cDNA synthesis

mRNAs were prepared from mouse testis using Quick Prep Micro mRNA purification kit (Pharmacia Biotech). Purified mRNAs were kept at -70° until ready used. First strand cDNA was synthesized from 1 μ g of mRNA using the First-Strand cDNA synthesis kit (Pharmacia Biotech). The human testis cDNA library was obtained from Clontech.

Polymerase chain reaction protocol

PCR was performed in a DNA thermal cycler with 5 μ l of cDNA template and 1 μ l of Taq DNA polymerase in a final volume of 100 μ l, containing 1 mM $MgCl_2$, 2 μ M of each primer oligonucleotide, 20 μ M of each dNTP and 5% DMSO. Cycling profiles included an initial denaturation step of 5 min at 94° C., followed by 30 cycles of 1 min at 94° C., 1 min at 40° C. and 1.5 min at 72° C. A final extension step was performed at 72° C. for 10 min. The amplified DNA was loaded on a 2% agarose gel and visualized by staining with ethidium bromide. Fragments ranging in size between 500–700 bp were cut and eluted from the

gel. If needed, a second round of PCR was done with nested oligonucleotide primers, using 10 μ l of the first PCR reaction, or of the eluted band cut from the agarose gel. Resulting fragments were ligated in pCR2.1 vector (Invitrogen) according to the distributor's recommendations. DH5 α *Escherichia coli* cells were transformed with the ligation mixture and grown on 2YT plates in the presence of kanamycin. Plasmids were prepared from resistant cells and sequenced.

In situ hybridization on mouse tissues and chromosomal localization of human genes

In situ hybridization on whole mouse slices or isolated tissues was performed as described previously (Ruchon et al., 1998).

To determine the chromosomal localization of human NL-2 and NL-3 genes, a technique for mapping genes directly to banded human chromosomes was used. Metaphase chromosomes were obtained from lymphocytes cultured from normal human peripheral blood. Cells were synchronized with thymidine and treated with 5-bromodeoxyuridine (BrdU) during the last part of the S phase to produce R-banding. Biotin-labeling of the probe was done by nick-translation (Bionick, BRL) and the probe was visualized by indirect immunofluorescence.

Antibody production

To raise antibodies against the new peptidases, the cDNA sequences of each protein was compared to that of other members of the family and the sequence segment showing the less homology was used. These sequences are from amino acid residues 273 to 354 for NL-1, from 75 to 209 for NL-2 and from 143 to 465 for NL-3. These cDNA fragments were cloned in vector pGEX2T (Pharmacia Biotechnology) downstream from and in phase with Glutathione-S-transferase (GST). Plasmids were transformed in *E. coli* strain AP401 and, induction of synthesis and purification of the fusion proteins were performed as recommended by the supplier. The NL polypeptides were cleaved from the fusion protein with thrombin and purified by SDS-PAGE. NL polypeptides were injected to rabbits or mice according to the following schedules: for rabbits, initial injection of 150 μ g of protein with boosts of the same amount 4 weeks and 8 weeks following the initial injection; for mice, initial injection of 100 μ g of protein followed by boosts of the same amounts 3 and 6 weeks later. A month after the last injection, sera were collected from the animals and tested by immunoblotting against the initial *E. coli*-produced antigens and the recombinant proteins produced in mammalian cell lines.

Production of monoclonal antibodies

cDNA fragments corresponding to amino acids segments of NLs selected to raise antibodies were used to construct a GST-fusion protein in *E. coli*. This fusion protein was purified from *E. coli* extracts by affinity chromatography on a glutathione-Sepharose column according to the supplier's instructions (Amersham-Pharmacia). After thrombin cleavage, the NL portion of the GST fusion protein was further purified by electroelution from a polyacrylamide gel. This material was used to immunise 4 mice (5 injections of \approx 50 μ g of NL polypeptide). Blood was collected from each mice after the immunisation schedule and the presence of antibodies in mice serum was assessed by ELISA using microtiter plates coated with NL polypeptide from *E. coli* extracts. Mice sera were also tested for the presence of NL antibodies by Western blotting extracts of mammalian cells transfected with the NL expression vectors. One mouse selected for its high titer of NL specific antibodies (as measured by ELISA)

was sacrificed and its spleen cells were collected and immortalised by fusion with myeloma cells (strain: P3-X63Ag.653 from ATCC) as described previously (Crine 1985). Hybridoma cells were selected for their ability to grow in HAT selection medium and cloned by several rounds of limiting dilution. Hybridomas showing proper affinity and specificity to the enzymes NL-1, NL-2 and NL-3 were selected.

Expression of NLs in cultured mammalian cells and enzymatic assays The cDNAs for NL-1 and NL-3 were cloned in vectors pcDNA3 or pRcCMV (Invitrogen) and introduced by transfection in mammalian cell lines according to procedures already described in our laboratory (Devault et al., 1988a). Procedures to prepare extracts of cellular proteins or culture media were also described in previous papers (Devault et al., 1988a; Lemay et al., 1989). The presence of NLs in these extracts was monitored by immunoblotting using specific antibodies.

Extracts of cellular proteins and culture media were assayed for enzymatic activity. Two tests were performed. The first used [³H]-Tyr-(D)Ala₂-Leu-enkephalin as substrate and was performed according to Lemay et al., (1989). The second used bradykinin as substrate and was performed as described by Raut et al. (1999).

Results

Cloning of NL-1, a new member of the NEP family

The molecular cloning in the past few years of ECEs, PEX and KELL showed that all these proteins have between 50 and 60% similarity with NEP. This observation led us to believe that these peptidases are part of an extended family and that there could be still additional members to be discovered. To test this hypothesis, we aligned the amino acid sequences of the members of the NEP-like family and designed degenerate oligonucleotide primers to be used in RT-PCR reactions (FIGS. 1 and 2). These primers were located on either side of the HEXXH consensus sequence for zincins. Because they are highly degenerate, primers 1 and 2 were each subdivided into two pools, 1A-1B, and 2A-2B, respectively (FIG. 2). Any PCR amplified DNA fragment that corresponds to a peptidase of the family should normally contain the consensus sequence and be easily recognized by sequencing of the cloned fragments. Using this strategy, we first performed PCR reactions with primer pairs 1A-3 and 1 B-3. The amplified DNA migrates mostly as a smear starting at around 700 bp and going down to 100 bp. As the expected fragments should be around 550 bp, we isolated from the gel the section corresponding to DNA fragments longer than 500 bp. A second round of PCR reactions was performed with both crude PCR products of the first reaction and isolated DNA bands, using primers 2A-3 and 2B-3. The expected 296 bp fragment was seen on the gel (not shown).

Cloning of these DNA fragments generated approximately 350 clones, of which 44 were sequenced. Nine of these had no inserts or corresponded to sequences not related to the NEP family, 24 corresponded to NEP, 3 to PEX, and 8 corresponded to one putative new member of the family, since they all contained the HEXXH consensus sequence for zincins and showed 65% homology with mouse NEP (in boxes FIG. 3). This fragment was then used to screen a mouse testis cDNA library, and allowed us to isolate a complete cDNA of 2592 nucleotides (FIG. 3). The identity of this sequence with other members of the family is presented in Table I. This new member was called NL-1, for NEP-like peptidase 1.

Cloning of NL-2 and NL-3

A strategy similar to that described for amplification of enzymes of the NEP family from mouse testis cDNAs was used with a human testis cDNA library using two different oligonucleotide primers. This time, DNA fragments of approximately 900 bp were obtained and cloned. Ten clones were sequenced, revealing the presence of NEP and two new peptidases of the family that we have called NL-2 and NL-3.

The NL-2 PCR fragment was 879 nucleotides in length and encoded a 293 amino acid residue segment probably located in the carboxy-terminal domain of this putative peptidase (in brackets FIG. 4). This PCR fragment was then used to screen a lambda gt10 human brain cDNA library. It allowed the isolation of other cDNA fragments which overlap partially with the NL-2 PCR fragment. Fusion of these lambda clones and the PCR fragment resulted in an open reading frame of 770 amino acid residues. The use of 5' RACE protocols with human testis cDNA libraries allowed completion of the sequence of NL-2 ORF (FIG. 4). This ORF codes for a putative protein that is about 80% identical to the mouse NL-1 protein (FIG. 6). Across species, members of the NEP, PEX, ECEs sub-families have highly conserved sequences (more than 94% identity). Although a sequence identity of about 80% only exists between the novel human protein and mouse NL-1, these proteins share unique characteristics that make possible the fact that NL-2 protein may be the human homologue of NL-1. The identity of NL-2 with other members of the family is presented in Table I.

The 879 bp PCR fragment encoding NL-3 showed an open reading frame of 293 amino acid residues (FIG. 5, in brackets). Sequence analysis of NL-3 showed that it was 94.2% identical to an EST sequence from mouse embryonic tissue present in publicly accessible DNA data banks. This mouse EST sequence, commercially available from American Tissue and Cell Culture (ATCC), had been obtained previously by our laboratories.

Since Northern blot analysis of human tissues with the NL-3 PCR fragment showed the expression of this protein in spinal chord (see below), the same PCR DNA fragment was used to screen by hybridization a human spinal chord cDNA library constructed in phage A vectors. One clone contained a full-length ORF of 752 amino acid residues that encompassed the 293 amino acid residue ORF of the PCR fragment. Further probing, cloning and sequencing lead to the obtention of NL-3 full sequence, shown in FIG. 5.

FIG. 6 presents a comparison of the amino acid sequence of the new NEP-like enzymes and Table I shows the extent of identity between members of the family.

Cellular distribution of NL-1, NL-2 and NL-3 peptidases

Determining the tissue distribution of NL-1, NL-2 and NL-3 may provide clues to identify the peptidergic systems in which they are involved. It will be particularly interesting to compare the tissue distribution of these peptidases with that of NEP and the ECEs to determine whether or not the physiological functions of NL-1 and/or NL-2 and/or NL-3 may overlap those of NEP and/or ECEs.

In situ hybridization (ISH), using our mouse cDNA, was used to determine the spatial and temporal expression of NL-1 during mouse development, as done previously for PEX (Ruchon et al., 1998)). Serial sections of whole foetal (12, 15 and 19 dpc) and adult mice (1, 3 and 6 days old) were hybridized with an [³⁵S]-labeled RNA probe. FIG. 7 shows a section of mouse testis which was the only tissue identified to express NL-1 by this technique. Cells of seminiferous tubules are specifically labeled but spermatids located near

the center of the tubule showed strongest labeling. These cells are in the last stage of maturation into spermatozooids. The presence of NL-1 in testis has now been confirmed by Northern analysis of mouse tissues (see FIG. 10). Other tissues express NL-1, when analyzed by RT-PCT, which is a more sensitive assay (not shown).

A similar approach was used to determine the localization of NL-3 using the mouse EST obtained from ATCC. FIG. 8 shows sections of whole mouse at 17 days of embryonic development and 4 days post-natal. Several tissues are expressing this putative peptidase including brain, where it is associated with neurons (FIG. 9), spinal chord, liver, spleen and bones. Labeling was stronger in bones from Hyp mouse, an animal model for hypophosphatemic rickets (FIG. 8). In bones, NL-3 was found to be expressed by osteoblasts (not shown).

Northern blotting experiments were performed on several tissues with NL-2 and NL-3 probes. A Human Multiple Tissues Northern Blot (Clontech) was hybridized with specific probes. A single RNA band of approximately 4.0 kb was revealed by the probe for NL-2. Expression of NL-2 is restricted to brain and spinal cord (not shown). However, RT-PCR has shown the presence of this enzyme in testis (not shown).

A single RNA band of approximately 3.0 kb was detected with the specific probe for NL-3 (not shown). NL-3 expression was observed mainly in ovary, spinal cord and adrenal gland.

Chromosomal localisation of the human gene for NL-2 and NL-3

As a mean to get clues on the function of the new metallopeptidases in vertebrates, we have localized the new cDNAs on human chromosomes, in order to look for a possible link between the gene locus and mapped genetic diseases in humans. To do so, we have mapped the NL-2 and NL-3 genes by high-resolution fluorescence in situ hybridization (FISH). NL-2 was localized to chromosome band 1p36. Consistent with the cellular distribution of NL-2 in humans, genetic diseases of the CNS such as dyslexia, neural tube defect, neuroblastoma, neuronal type of Charcot-Marie-Tooth disease have all been mapped in this region and represent potential targets for a role of NL-2 in humans. NL-3 was localized to chromosome band 2q37. Consistent with a role of NL-3 in bones, a form of Albright hereditary osteodystrophy was mapped to the same chromosomal locus (Phelan et al., 1995).

In view of the foregoing, NL-2 and NL-3 are metallopeptidases that are assumed to be immediately useful as markers for a disease or disorder associated with human chromosomal locus 1p36 and 2q37, respectively. Their localization on a chromosome band associated with known diseases suggests that they may be expressed or co-expressed with one or more genes, as a cause or a consequence of disease development. It is possible that these enzymes are up or down regulated, alone or along with other genes involved in a disease. Therefore, antibodies or other ligands specific to NL-2 or NL-3 may be used for a diagnostic purpose, as well as primers or probes in diagnostic assays using nucleic acid hybridization or amplification techniques. Otherwise, primers or probes directed against the nucleic acids of NL-2 and NL-3 would be useful to map the mutations of a gene located in close proximity and involved in the disease. Therefore, no matter which exact function NL-2 and NL-3 gene products have, their chromosomal localization provides one diagnostic utility. This localization as well as the tissular distribution

provide information as to the disease and tissue to be investigated to elucidate the exact function of these enzymes.

NL-1 resembles NL-2, sharing with the latter about 80% homology in the amino sequence and sharing structural characteristics such as the furin recognition sequence located at the proximal end of the ectodomain. NL-2 might be the human homologue of mouse NL-1. If such was the case, these two proteins would have a substantial degree of divergence and, maybe, different profiles of activity varying from one species to another.

Chromosomal localization of NL-1 was determined in mouse genome by Single Strand Conformational Polymorphism (SSCP) in collaboration with The Jackson Laboratory Backcross DNA Panel Mapping Resource. NL-1 was localized to the distal region of mouse chromosome 4 which corresponds to human chromosome region 1p36 where is located NL-2 gene. This reinforces our hypothesis that NL-1 and NL-2 are species variants.

Production of antibodies against NLs

Antisera collected from injected animals were first tested by immunoblotting on GST-antigen fusion proteins produced in *E. coli*. Antiserum from one rabbit recognized the NL-1 related polypeptide and antisera from one mouse and one rabbit reacted with the NL-3-related polypeptide (results not shown). The anti NL-1 antiserum and the mouse anti NL-3 antiserum, which appeared more specific than the rabbit antiserum, were next tested by immunoblotting on extracts of proteins and culture media from cells expressing NL-1 or NL-3 (see below).

Expression of NL-1 in CHO cells

The cDNA encoding the full-length NL-1 protein was cloned in the mammalian expression vector pcDNA3-RSV and transfected in CHO cells. Stable cell lines were established by selection with the drug G418 and tested by immunoblotting for the presence of NL-1.

Small amounts of NL-1 were found in the extracts of transfected CHO cells (results not shown). This intracellular species was sensitive to endo H digestion, indicating that the sugar moiety was not mature and suggesting ER localization (results not shown). The culture medium of transfected CHO cells showed the presence of soluble NL-1 (FIG. 10). This extracellular species was resistant to endo H suggesting true transport through the late secretory pathway. The cDNA sequence of NL-1 predicts a type II transmembrane protein. The mechanism by which NL-1 is transformed into a soluble protein is not known presently. However, examination of the amino acid sequence revealed the presence of a putative furin cleavage site from residue 58 to 65 (FIG. 3). A similar site is present in NL-2 sequence.

The soluble form of NL-1 was assayed for activity using [³H]-Tyr-(D)Ala₂-Leu-enkephalin and bradykinin as substrates. FIG. 11 shows that NL-1 can degrade the enkephalin substrate ($K_m=18\pm 10 \mu M$) and that this activity can be inhibited by phosphoramidon ($IC_{50}=0.9\pm 0.3 nM$) and thiorphan ($K_m=47\pm 12 nM$), a general inhibitor of enzymes of the NEP family. Bradykinin is also a substrate for NL-1 (not shown).

Use of NL-1 amino-terminal domain to promote secretion

The observation that NL-1 ectodomain was secreted, possibly through cleavage of the transmembrane segment by furin, raised the possibility to promote secretion of exogenous proteins that could be spliced to NL-1 amino-terminal domain (from initiator methionine to the furin site). To test this hypothesis, the ectodomain of NL-3 (from the third

cysteine to the end) was spliced to NL-1 amino-terminal domain using a PCR strategy and the recombinant DNA cloned in expression vector pRcCMV. The fusion protein was expressed by transfection of the vector in COS-1 and HEK 293 cells. The culture media of transfected cells was analyzed by immunoblotting using the mouse antiserum against NL-3. FIG. 12 shows the presence of NL-3 in the spent culture media of both COS-1 and HEK 293 cells. This result shows that NL-1 amino-terminal domain can be used to promote secretion of exogenous proteins.

The soluble form of NL-3 was assayed for activity using [³H]-Tyr-(D)Ala₂-Leu-enkephalin as substrate. No activity was found.

The previous experiment showed that it was possible to use the amino-terminal domain of NL-1 to promote secretion of an otherwise membrane attached protein ectodomain. To verify whether the same strategy could be used to promote secretion of small peptides, a PCR strategy was used to splice human β-endorphin to the amino-terminal domain of NL-1 and the recombinant DNA was cloned in vector pRcCMV. The fusion protein was expressed by transfection of the vector in COS-1 and HEK 293 cells. The culture media of transfected cells was collected 48 h after transfection and the peptides purified as described previously (Noël et al., 1989). The presence of β-endorphin in the extracts was detected by radioimmunoassay. The results showed that both COS-1 and HEK 293 cells produced approximately 100 pg of β-endorphin per ml of culture medium. Therefore, the N-terminus of LN-1 and NL-2 which ends with a furin-recognition site will be useful to produce the soluble form of a protein of interest.

Section 2)

Materials and Methods

DNA manipulations

All DNA manipulations, phage library screening, and plasmid preparations were performed according to standard protocols (Ausubel 1988; Sambrook 1989). Site-directed mutagenesis was performed using a PCR-based strategy as described previously (Le Moual 1994).

mRNA purification and RT-PCR protocol for identification of new members of the neprilysin family

mRNAs were prepared from mouse testis using Quick Prep Micro mRNA purification kit (Pharmacia Biotech). First strand cDNA was synthesized from 1 μg of mRNA using the First-Strand cDNA synthesis kit (Pharmacia Biotech).

Two sense primers, oligonucleotide 3817 (5'-TGGATG-GAT/CGA/CIGG/AIACIA/CA-3') and oligonucleotide 3719 (5'-A/GTIGTITTT/CCCIGCIGGIA/GT/AIC/TTG/CCA-3') corresponding respectively to amino acid residues 459 to 465 and 552 to 560 of NEP sequence, and one antisense primer, oligonucleotide 3720 (5'-AIICCICIA/TC/TA/GTCIGCIG/AC/TA/GTTT/CTC-3') corresponding to amino acid residues 646 to 654 (see FIGS. 1 and 2), were synthesized. PCR was performed with 5 μl of cDNA template and 1 μl of Taq DNA polymerase in a final volume of 100 μl, containing 1 mM MgCl₂, 2 μM of each oligonucleotide 3817 and 3720, 200 μM of each dNTP and 5% DMSO. Cycling profiles included an initial denaturation step of 5 min at 94° C., 30 cycles of 1 min at 94° C., 1 min at 40° C. and 1.5 min at 72° C., and a final extension step at 72° C. for 10 min. One half of the amplified DNA was fractionated on a 2% agarose gel and fragments ranging in size between 500–700 bp were purified and resuspended in a final volume of 50 μl. A second round of PCR was done with primers

3719 and 3720, using as template either 10 μl of the first PCR reaction or 5 μl of the purified fragments, and the new PCR products were ligated in pCR2.1 vector (Invitrogen). Several identical clones corresponded to a potential new member of the NEP family. We called this member NL1 for NEP-like 1.

Cloning of full-length NL1 cDNA

The cloned NL1 PCR fragment was used as probe to screen a mouse testis λ Uni-ZAP™XR cDNA library (Stratagene). Twelve out of a hundred positive phages were plaque purified and subcloned into pBS SK vector (Stratagene). As the longest clone analyzed presented an incomplete ORF (pBS-NL1A), 5'RACE with primers located in vector (5'-TAGTGGATCCCCCGGGCTGCAG-3', sense primer) and NL1 (5'-ACCAAACCTTTCCTGTAGCTCC-3', antisense primer, nt 1303 to 1324 of NL1; was subsequently performed on the DNA of the remaining semi-purified positive clones. Amplification was performed with 1 μl of Vent polymerase in a final volume of 100 μl containing 50 ng of DNA, 4 mM of MgSO₄, 1 μM of each oligonucleotide, 200 μM of each dNTP and 10% DMSO. Cycling parameters included an initial denaturation step of 1 min at 94° C., 25 cycles of 30 sec at 94° C., 30 sec at 60° C. and 1 min at 72° C., and an incubation of 10 min at 72° C. A PCR fragment of the expected length was subcloned into pCR2.1 vector (clone pCR-NL1A), but sequencing revealed no initiator ATG codon. A nested 5'RACE was then performed on mouse testis cDNA using the Marathon Ready cDNA kit (Clontech) with sense oligonucleotides AP1 and AP2 (from the kit) and NL1 antisense oligonucleotides 5'-CCTGAGGGCTCGTTTTACAACCGTCCT-3' (nt 503 to 529 of NL1) and 5'-CTCATCCCAGGAGAAGTGTAG-CAGGCT-3' (nt 475 to 502 of NL1) as recommended by the supplier. The resulting fragment was cloned into pCR2.1 vector (pCR-NL1B). Since only ten bp were missing for the initiator ATG codon, we reconstructed the 5' end of the cDNA by PCR-amplifying clone pCR-NL1A with sense primer 5'-CCACC
ATGGTGGAGAGAGCAGGCTGGTGTTCGGAAGAAG-3' (nt 332 to 364 of NL1; the 10 missing nucleotides are underlined) and antisense primer 5'-ACCAAACCTTTCCTGTAGCTCC-3' (nt 1303 to 1324 of NL1) using Vent polymerase as described above. The DNA fragment was then inserted into pCR2.1 (clone pCR-NL1C). The entire ORF was reconstituted following digestion of pBS-NL1A and pCR-NL1C with EcoRI and PflMI. The 5' end of NL1 cDNA was excised from pCR-NL1C and ligated into pBS-NL1A at the corresponding sites, resulting in plasmid pBS-NL1B. For expression studies, a BamHIIApal fragment generated out of pBS-NL1B, corresponding to the full length cDNA of NL1, was inserted into pCDNA3/RSV [18] vector.

Production of polyclonal antibodies

A plasmid for the production in *Escherichia coli* of a GST fusion protein with NL1 was constructed using pGEX-4T-3 expression vector (Pharmacia Biotechnologies). A 255 bp fragment from NL1 was amplified by PCR with Vent polymerase using sense primer 5'-GCTACG
GGATCCCGTGGCCACTATGCTTAGGAA-3' (nt 1139 to 1158) and antisense primer 5'-CGATTG
CTCGAGTGGGMCAGCTCGACTTCCA-3' (nt 1377 to 1396). Both pGEX-4T-3 and the PCR product were digested with BamHI and XhoI and ligated. The recombinant protein was produced and purified according to the supplier's instructions. Five weeks old female balb/c mice were immunized at monthly intervals for 3 months with 20 μg of the

recombinant NL1 fragment in Freund's adjuvant and antisera were subsequently collected.

Cell culture and transfection

Human Embryonic Kidney (HEK 293) cells were maintained in DMEM medium containing 10% fetal bovine serum (FBS), and supplemented with penicillin at 60 $\mu\text{g}/\text{ml}$, streptomycin at 100 $\mu\text{g}/\text{ml}$ and fungizone at 0.25 $\mu\text{g}/\text{ml}$. Transfections of cells with appropriate plasmids were performed by the calcium/phosphate-DNA co-precipitation method (Chang 1987). To establish permanent cell lines, G418 selection was initiated 48 h after the transfections at 400 $\mu\text{g}/\text{ml}$ for 12 days and gradually decreased at 100 $\mu\text{g}/\text{ml}$.

LLC-PK₁ cells transfected with pRcCMV-sNEP were maintained as described previously (Lanctot 1995).

Immunoblot analysis

For immunoblot analysis, cells were incubated for 16 h in synthetic DMEM medium containing 2 mM sodium butyrate. Cellular proteins were solubilized as previously described (Dion 1995). Secreted proteins recovered in culture media were concentrated approximately 10 fold by ultrafiltration. Immunoblot analysis were performed using the NEN Renaissance kit with the polyclonal antibody specific to NL1 or the α 1-antitrypsin inhibitor antibody (Calbiochem; LaJolla, Calif.) followed by the appropriate horseradish peroxidase-conjugated IgG (Vector Laboratories).

For the glycosylation studies, proteins were incubated with endoglycosidase H (endoH) or peptide:N-glycosidase (PNGaseF) as suggested by the distributor (NEB).

Enzymatic activity assays

NL1 activity was monitored and compared to sNEP activity using (Tyrosyl-[3,5-³H])(D-Ala₂)-Leu₅-enkephalin (50 Ci/mmol) (Research Products International Inc.), as already described (Dion 1995; Devault 1988). K_m values were determined by the isotope-dilution method. The inhibitory effects of phosphoramidon and thiorphan were also assessed as previously described (Dion 1995).

HPLC analysis of the hydrolysis of Leu-enkephalin

Five μg of Leu₅-enkephalin were incubated at 37° C. for one hour in 50 mM MES, pH 6.5, with concentrated culture medium of HEK 293 cells expressing NL1 (~300 μg of total proteins) or LLC-PK₁ cells expressing sNEP (~30 μg of total proteins), in absence or presence of 0.1 mM phosphoramidon. Hydrolysis products were separated by reversed-phase HPLC as described previously [23]. Tyr-Gly-Gly and Phe-Leu were both identified by elution profiles of synthetic marker peptides.

Northern blot analysis

A mouse multiple tissue poly(A)⁺ mRNA blot (Clontech) was hybridized with a [³²P]dCTP random primer labelled probe in ExpressHyb solution (Clontech). The blot was washed according to the manufacturer's recommendations and exposed to Fuji RX film for 7 days at -80° C. with intensifying screens.

RT-PCR screening of mouse tissues

First strand cDNA synthesis was performed with 1 μg of total RNA from mouse tissues and oligo(dT) as primer, using Gene Amp RNA PCR Core Kit (Perkin Elmer). For the PCR reactions, primers 5'-TGGCGAGAGTGTGTCAGCTATGTC-3' and 5'-CTTCCAAAATGTAGTCAGGGTAGCAATC-3' were used with Taq polymerase. One tenth of the PCR products were visualized on a 4% agarose gel.

In situ hybridization

To construct a plasmid for the synthesis of cRNA probes for ISH, pCR-NL1A was used as template to amplify a 452 bp fragment by PCR with sense primer 5'-GGAGCCAT-AGTGACTCTGGGTGTC-3' (nt 416 to 439) and antisense primer 5'-GACGCTCAGCAGGGGCTCAGAGTC-3' (nt 842 to 865). The amplification product was inserted into pCRII vector (Invitrogen). Synthesis of riboprobes and protocols for ISH were as described previously (Ruchon 1998).

Results

Cloning and sequence analysis of mouse NL1 cDNA

In order to isolate cDNAs for new members of the NEP family, we developed an RT-PCR strategy based on fact that NEP, ECE-1 and PHEX share regions of significant sequence identity. Following RT-PCR on testis mRNAs with nested primers, a DNA fragment of approximately 300 bp was amplified. This DNA fragment was cloned and the plasmids from 24 independent colonies were sequenced: 3 clones had no insert, 4 clones had DNA fragments not related to the NEP family, 7 clones had sequences corresponding to mouse NEP and 3 clones had sequences corresponding to mouse PHEX, showing that our approach efficiently allowed the identification of members of the family. Moreover, 7 identical clones had a new cDNA presenting sequence similarities to members of the NEP family. The full-length cDNA was subsequently obtained by screening a mouse testis A cDNA library followed by 5'RACE, as described under Materials and Methods. Its nucleotide and deduced amino acid sequences confirm that we cloned a novel NEP-like protein, referred to thereafter as NL1.

NL1 cDNA spans 2925 nt, including a 5'-untranslated region of 331 nt, an open reading frame of 2295 nt from nt 332 to nt 2626, and a 3'-untranslated region of 299 nt. The sequence surrounding the proposed initiator ATG conforms to the Kozak consensus (Kozak 1986). The deduced amino acid sequence of NL1 reveals a putative type 11 transmembrane protein of 765 amino acid residues encompassing a short N-terminal cytoplasmic tail, a unique putative transmembrane domain, and a large C-terminal extracellular domain. The ectodomain contains nine potential N-glycosylation sites (Asn-X-Ser/Thr) and ten cysteine residues corresponding to those conserved among all the members of the family, which are presumably involved in proper folding and in maintenance of the protein in an active conformation. All amino acid residues known to be part of the active site of NEP are present in NL1. The predicted protein presents greater similarities to NEP than to any other member of the family. Although NL1 shares numerous features with proteins of the neprilysin family, a notable aspect distinguishes it from the others: the first conserved cysteine residue of the ectodomain is more distant (34 amino acid residues) from the predicted transmembrane domain in NL1 than it is in NEP (9 residues) or any other members of the family. Moreover, we noticed a putative furin cleavage site (-Arg₅₈-Thr-Val-Val-Lys-Arg₆₃-) between the end of the transmembrane domain and the first cysteine. This observation suggests that NL1 could exist as a secreted rather than a membrane-bound protein.

NL1 expression in HEK 293 cells

HEK 293 cells were transfected with pCDNA3/RSV expression vector containing NL1 cDNA, and a permanent cell line was established as described under *Materials and Methods* (HEK/NL1 cells). Immunoblotting with a polyclonal antibody showed that after 16 h of culture, most NL1

was present in the culture medium with small amounts of the enzyme in the cell extract. Secreted and cell-associated NL1 had apparent molecular masses of approximately 125 and 110 kDa, respectively. To characterize the glycosylation state of NL1, we next submitted the recombinant protein to deglycosylation by peptide:N-glycosidase F (PNGase F) and endoglycosidase H (endo H). PNGase F removes high mannose as well as most complex N-linked oligosaccharides added in the Golgi complex. In contrast, endo H removes N-linked oligosaccharide side chains of the high mannose type found on proteins in the RER but which have not yet transited through the Golgi complex; thus, resistance to endo H can be used as an indication that the protein has traveled through the Golgi complex. PNGase F treatment showed that the cell-associated and secreted NL1 were N-glycosylated as their electrophoretic mobility increased following digestion. However, the secreted NL1 migrated as a doublet after PNGase F treatment, with one band co-migrating with cell-associated form and the second having a slower rate of migration. Since untreated and endo H-digested secreted NL1 are seen as single bands by SDS-PAGE, our observation suggests that a proportion of secreted NL1 undergoes further post-RER postranslational modification that renders some of the N-linked oligosaccharides resistant to PNGase F digestion.

In contrast to secreted NL1, NL1 from cell extract was sensitive to endo H treatment. This result shows differences in the glycosylation state of the two species and suggests that the cell-associated form observed in transfected cells is an intracellular species that has not traveled through the Golgi complex.

Processing of NL1 by a subtilisin-like convertase

To determine whether a member of the mammalian subtilisine-like convertase family is responsible for NL1 presence in the culture medium of transfected cells, we co-transfected transiently HEK 293 cells with a constant amount of plasmid pCDNA3/RSV/NL1 and increasing amounts of plasmid pCDNA3/CMV/PDX (Benjannet 1997). This latter vector promotes the expression of the α 1-antitrypsin Portland variant, α 1-PDX, a known inhibitor of subtilisin-like convertases (Anderson 1993). Immunoblot analysis of the culture media of cells expressing both NL1 and α 1-PDX indicated that NL1 secretion was strongly inhibited by the presence of α 1-PDX: a relation was observed between the amounts of α 1-PDX and the level of inhibition of NL1 secretion.

To confirm that proteolysis by the subtilisin-like convertase occurred at the putative furin cleavage site identified in NL1 ectodomain (-Arg₅₈-Thr-Val-Val-Lys-Arg₆₃-), the amino acid residues Asn₆₂-Gly₆₃ were substituted for Lys₆₂-Arg₆₃ by site-directed mutagenesis in vector pCDNA3/RSV/NL1 and the mutated vector used to establish HEK 293 cells expressing the mutant protein (HEK/NL1mut cells). Immunoblot analysis of the culture media of HEK/NL1mut cells showed that the mutation totally abolished secretion of NL1. Furthermore, an additional form of NL1 with a molecular mass of 127 kDa was detected in the extract of these cells. This new species was resistant to endo H digestion and was found associated with membranes when HEK/NL1mut cells were fractionated according to Chidiac et al. 1996 (result not shown).

NL1 enzymatic activity

Culture media from HEK 293 and HEK/NL1 cells were tested for enzymatic activity using as substrate (Tyrosyl-[3, 5-³H](D-Ala₂)-Leu₅-enkephalin, a known NEP substrate. Activity was detected in the culture medium of HEK/NL1

cells but not in that of HEK 293 cells. This activity increased linearly with the amounts of NL1 and with the incubation period, indicating that degradation of the substrate was due to NL1 enzymatic activity.

We next characterized NL1 enzymatic parameters using the same substrate and compared them to those of an engineered soluble form of NEP (sNEP) (Lemay 1989). NL1 affinity for D-Ala₂-Leu₅-enkephalin was slightly higher than that of sNEP as shown by their K_m values of 18 μ M and 73 μ M, respectively. Inhibition assays showed that phosphoramidon had similar effects on NL1 and sNEP activity, with IC_{50} values of 0.9 nM and 0.5 nM respectively, and that thiorphan, a specific inhibitor of NEP, inhibited NL1 with an IC_{50} of 47 nM, as compared with an IC_{50} of 8 nM for NEP.

Very low levels of phosphoramidon-sensitive activity was detected in extracts of HEK/NL1 cells (data not shown) consistent with the small amounts of NL1 observed by immunoblotting.

To determine whether NL1 had cleavage site specificity similar to NEP, we incubated Leu₅-enkephalin in the presence of NL1 recovered from the medium of HEK/NL1 cells or in the presence of sNEP, and analyzed the degradation products by RP-HPLC. Peaks co-migrating with standard Tyr-Gly-Gly and Phe-Leu peptides were observed in both RP-HPLC profiles, indicating that both enzymes cleaved the substrate at the Gly₃-Phe₄ peptide bond. This enkephalin-degrading activity was completely inhibited by phosphoramidon (1 μ M).

Tissue and cellular distribution of NL1 mRNA

Tissue distribution of NL1 mRNA was determined by Northern blot analysis with a specific probe corresponding to the 5'end of the coding region of NL1 cDNA. A single transcript of 3.4 kb was detected exclusively in testis among all the mouse tissues tested. Mouse tissues were also screened by RT-PCR. Using this more sensitive technique, expression of NL1 was observed in several other tissues including heart, brain, spleen, lungs, liver and kidney. Consistent with the Northern blot results, RT-PCR analysis, although not strictly quantitative, detected more NL1 mRNA in testis than in other tissues.

To gain more insight into NL1 mRNA distribution, we examined by ISH cryostat sagittal sections from a 4-day newborn mouse, as well as sections from a 16-day old animal (p16) and adult tissues (heart, brain, spleen, lungs, liver, kidney and testis). The presence of NL1 mRNA was detected only in adult testis. Only the germinal cells in the luminal face of the seminiferous tubules were labeled. These cells were identified as round and elongated spermatids in all spermiogenesis maturational stages. Neither spermatozoa nor spermatocytes, spermatogonies or Sertoli cells were labeled. Interstitial cells were also negative. Controls were performed with sense riboprobes, which produced only nonspecific background (data not shown). The 4-day old mouse sagittal sections and all other tissues tested were negative.

Discussion

The great interest in members of the Neprilysin family as putative therapeutic targets, and the recent discovery of new members of this important family of peptidases led us to investigate whether additional members of the family remained to be identified. Using a PCR-based strategy, we cloned, from mouse testis, a partial cDNA encoding a new NEP-like enzyme that we called NL1. Analysis of the amino acid sequence encoded by the full-length NL1 cDNA revealed that this member of the family resembles NEP the most: 55% identity and 74% similarity. Recently, the pri-

mary structure of a new zinc metallopeptidase from total mouse embryo was reported (Ikeda 1999). This enzyme, called SEP, is found either as a soluble or a cell-associated form due to alternative splicing. NL1 shows only 3 amino acid differences with the soluble form of SEP indicating that secreted SEP and NL1 are the same enzyme. Our cloning strategy did not allow characterization of the cell-associated form of NL1 which is a minor species in mouse testis (Ikeda 1999).

The amino acid sequence of NL1 predicts a topology of a type II integral membrane glycoprotein that is similar to the other members of the family. Treatment of the recombinant protein with PNGase F showed that indeed NL1 possesses N-linked carbohydrate side chains. However, it is not possible to determine precisely whether all nine putative N-glycosylation sites are used, but the 30 kDa decrease in molecular mass upon PNGase F treatment suggests that most are glycosylated. It has already been shown that all asparagine residues in a Asn-X-Ser/Thr consensus are glycosylated in rabbit NEP expressed in COS-1 cells and that sugar moieties increase the stability and enzymatic activity of the protein and facilitate its intracellular transport (Lafrance 1994). Three of NEP glycosylated Asn residues (Asn 145, Asn 285 and Asn 628) are conserved in NL1 (Asn 163, Asn 303 and Asn 643). Amongst these residues, Asn 145 and Asn 628 have been reported to influence NEP enzymatic activity (Lafrance 1994). In the same work, it has also been shown that the effect of sugar addition on folding and intracellular transport of NEP is a cumulative effect of all glycosylation sites rather than a contribution of any particular one. Glycosylation of NL1 may share similarities with that of NEP since we found their primary structures and enzymatic activities to be very similar.

Surprisingly, expression of the cDNA by transfection of HEK 293 cells showed that most of the enzyme was secreted in the culture medium. The small amount of NL1 associated with the cells was endo H-sensitive, suggesting that the cell-associated enzyme is a species that has not yet left the RER. The presence of a furin cleavage site in NL1 sequence between the predicted transmembrane domain and the first conserved cysteine residue of the ectodomain led us to believe that a member of the mammalian subtilisin-like family of convertases was responsible for the presence of NL1 in the culture medium. These enzymes are involved in processing a variety of precursor proteins such as growth factors and hormones, receptors, plasma proteins, matrix metalloproteinases, metalloproteases-desintegrins and viral envelope glycoproteins [for a review see: (Nakayama 1997)]. Site-directed mutagenesis of the furin cleavage site (-Arg₅₈-Thr-Val-Val-Lys-Arg₆₃-) and expression of α 1-PDX, a potent inhibitor of mammalian subtilisin-like convertases (Anderson 1993), confirmed that a member of this family of endoproteases was involved in NL1 secretion presumably by cleaving in carboxy-terminus of Arg₆₃. There are only a few examples of proteins which are processed from a membrane-bound precursor to a secreted form following cleavage by subtilisin-like convertases; these include meprin and collagen XVII (Milhiet 1995; Schacke 1998). Three members of the subtilisin-like family of convertases, namely furin, PC4 and PC7, are known to be expressed in germ cells (Nakayama 1992; Torri 1993; Seidah 1992, 1996). Whether one of these convertases generates secreted NL1 from its membrane form is under current investigation. In any case, NL1 is the only known member of the neprilysin family that is secreted. This unique feature suggests that NL1 plays its physiological role in a context different from that of the membrane-bound peptidases, thereby diversifying the role

of the peptidases of the neprilysin family. It is of interest that circulating forms of NEP in blood and urine have been described, but they have generally been related to pathological or stressful conditions (Almenoff 1984; Deschodt-Lanckmann 1989; Johnson 1985; Soleilhac 1996; Aviv 1995).

We have observed in cells expressing NL1 mutated at the furin cleavage site the appearance of a species resistant to digestion by endo H. This mutated protein was associated with cellular membranes. Taken together, these results indicate that NL1 is first synthesized and inserted in the RER membrane as a type II transmembrane protein. During intracellular transport, NL1 is converted to a soluble form by the action of a member of the mammalian subtilisin-like convertases. The identity of the cellular compartment where this process occurs is not known. However, mammalian subtilisin-like convertases are usually active in post-Golgi compartments of the secretory pathway suggesting that processing of NL1 from the membrane bound form to the soluble form is a post-Golgi event.

Despite almost total abrogation of NL1 secretion, we observed only a slight accumulation of endo H-resistant NL1 in cells either co-expressing α 1-PDX and NL1 (result not shown) or expressing mutated NL1. This observation suggests that unprocessed NL1 is rapidly degraded. A similar behavior was reported for the Notch1 receptor expressed in the furin-deficient cell line LoVo (Logeat 1998). The mechanism(s) by which these unprocessed proteins are degraded is still unknown. It is interesting to point out that the spliceform of SEP that has lost a 23 amino acid peptide, including the furin cleavage site, generates a cell-associated endo H-sensitive molecule (Ikeda 1999).

The most important observation regarding the NL1 primary structure is the conservation of residues which in NEP are essential for catalysis and binding of substrates or inhibitors. This finding suggests that NL1 could effectively act as an endopeptidase with a catalytic mechanism similar to that of NEP. This hypothesis was supported by the demonstration that D-Ala₂-Leu₅-enkephalin, a peptide substrate often used to monitor NEP activity, was also an excellent NL1 substrate. The affinity of NL1 for D-Ala₂-Leu₅-enkephalin was even higher than that of NEP, as reflected by a K_m value 4- to 5-fold lower. Furthermore, two well known NEP inhibitors, phosphoramidon and thiorphan, also abolished NL1 activity. Phosphoramidon, which inhibits NEP as well as ECE-1 activity, albeit to a lesser extent (Turner 1996), had very similar effects on NL1 and NEP, with an IC₅₀ value for NL1 varying not more than two-fold from the value determined for NEP. Thiorphan, considered to be a more specific inhibitor of NEP, also inhibited NL1 activity, with an IC₅₀ six-fold greater than that for NEP. These results suggest that the active sites of NL1 and NEP are similar. This hypothesis is supported by the observation that secreted SEP degraded a set of peptides known to be NEP substrates, including substance P, bradykinin and atrial natriuretic peptide (Ikeda 1999). Taken together, these results illustrate the importance of identifying and characterizing other member of the family for the design of highly specific inhibitors.

In agreement with the enzymatic parameters demonstrating that NL1 and NEP have similar catalytic sites, we have observed that both enzymes cleaved Leu₅-enkephalin at the same peptide bond. This result suggests that NL1 hydrolyzes peptide bonds on the amino side of hydrophobic amino acid residues as does NEP (Turner 1985). However, several other peptides will have to be tested to confirm this specificity and to determine whether NL1 has dipeptidyl carboxypeptidase

activity as was shown for NEP (Malfroy 1982; Bateman 1989; Beaumont 1991) and more recently for ECE-1 (Johnson 1999).

RT-PCR experiments with specific primers for the soluble and cell-associated forms of SEP showed a wide tissue distribution of the enzyme with the soluble form of SEP being predominant in testis and the cell-associated form in other tissues (Ikeda 1999). Our RT-PCR results confirmed the wide tissue distribution of NL1. However, Northern blotting and in situ hybridization experiments indicated that expression of NL1 is predominant in germ cells of mature testis. Interestingly, proenkephalin mRNA has been shown to be expressed in germ cells and somatic cells of the testis (Torii 1993, Seidah 1992; Kew 1989; Mehta 1994; Kilpatrick 1986, 1987). Specific functions for testicular enkephalin peptides have not yet been defined, but it is believed that they could act as intratesticular paracrine/autocrine factors. In addition to their putative role as mediators of testicular cell communication, it has also been demonstrated that proenkephalin products synthesized by spermatogenic cells during spermatogenesis are stored in the acrosome of human, hamster, rat and sheep spermatozoa and are released from sperm following acrosomal reaction (Kew 1990). It has thus been proposed that proenkephalin products may act as sperm acrosomal factors during the fertilization process as well as intratesticular regulators secreted by spermatogenic cells. Since Leu₅-enkephalin was found to be a good substrate for NL1, opioid peptides originating from proenkephalin could serve as physiological substrate for this new enzyme. In this way, NL1 would serve to regulate the activity of these bioactive peptides.

Testis is the only tissue where the soluble form of SEP is predominant (Ikeda, 1999), suggesting a testis-specific alternative splicing. Expression of testis-specific molecular species of peptidases or prohormones, arising through diverse mechanisms, has been documented in the past (Howard 1990; Jeannotte 1987). However, the physiological significance of these testis-specific species is not always clear. In the case of NL1 or SEP, it might allow local constitutive secretion by germinal cells of an otherwise cell-associated enzyme, to regulate spermatogenesis much like several other proteolytic enzymes of the seminiferous tubules (Monsees 1998). Alternatively, it might allow accumulation in acrosome with proenkephalin peptides and release upon acrosomal reaction. More exhaustive studies concerning NL1 localization and physiological substrate identification will be needed to understand its role in the testis and possibly in the fertilization process.

Cloning of other members of the family

To find other members of the NEP-like family, we will use the same RT-PCR strategy to amplify mRNA isolated from tissues known to be regulated by peptidergic systems (brain, thymus, kidney, heart, lung, ovary, pancreas, bone, bone marrow and lymphoid cells). In fact, many of these tissues are known to express at least one member of the family and/or to control a peptidergic pathway on which peptidase inhibitors have major effects. Amplified fragments will be cloned and the resulting clones will be sequenced and compared to the sequence of known peptidases, as described above. Pairs of degenerate primers in other highly conserved regions will also be designed to increase the possibility of cloning other relevant peptidases.

DISCUSSION

As discussed above, peptidases of the NEP family known to date have often been found to play important physiologi-

cal roles. This is certainly true for NEP itself, ECEs and PEX, (see review above). For this reason, some of these enzymes (as it was the case for NEP and ECE in the past) might be interesting targets for the design of inhibitors that in turn could be used as therapeutic agents in various pathological conditions. However, it is of some concern that inhibitors designed for one enzyme may also inhibit to some extent other members of the family. This lack of specificity for an inhibitor used as a therapeutic agent in the long term treatments such as those used as antihypertensive agents for instance, may cause unforeseen problems due to unwanted side effects. The objectives of the present work was to develop a strategy to clone new members of the NEP family of peptidases. The results presented in this report clearly show that our strategy can be successful. We have determined the complete or partial nucleotide sequence of three cDNAs encoding putative enzymes of the NEP family.

These cDNA sequences are valuable tools and may be used to:

Produce antibodies

As shown in the present work, knowledge of NL cDNA sequences can be used to raise specific antibodies. For example but not exclusively, regions of less homology between the peptidases (amino acid residues 50 to 450) can be used to synthesize peptides whose sequences are deduced from the translation of the cDNAs, and/or bacterially-expressed fragments of the cDNAs fused for example but not exclusively to GST may be purified and injected into rabbits or mice for polyclonal or monoclonal antibody production. These antibodies can be used to:

identify by immunohistochemistry the peptidergic pathways in which the peptidases are functioning;

study the physiopathology of NL-enzymes by immunoblotting or immunohistochemistry on samples of biological fluids or biopsies;

set up high through put screening assays to identify NL-enzymes inhibitors. This can be done for example but not exclusively by using the antibodies to attach the NL-enzymes to a solid support;

purify NL-enzymes with said antibodies by immunoprecipitation or affinity chromatography by identifying antibodies capable of selectively binding to the NL-enzymes in one set of conditions and releasing it in another set of conditions typically involving a large pH or salt concentration change without denaturing the NL-enzyme;

identify antibodies that block NL-enzymes activities and use them as therapeutic agents. Blocking antibodies can be identified by adding antisera or ascite fluid to an in vitro enzymatic assay and looking for inhibition of NL-enzymes activities. Blocking antibodies could then be injected to normal or disease model animals to test for in vivo effects.

Derive specific RNA or DNA probes

As shown in the present work, knowledge of the nucleotide sequence of the members of the NEP-family allows nucleotide sequence comparisons and facilitate the design of specific RNA or DNA probes by methods such as but not exclusively molecular cloning, in vitro transcription, PCR or DNA synthesis. The probes thus obtained can be used to:

derive specific probes or oligonucleotides for RNA and DNA analysis, such as Northern blot and in situ hybridization, chromosome mapping or PCR testing.

These probes could be used for genetic testing of normal or pathological samples of biological fluids or biopsies;

make vectors for gene knock-out or knock-in in mice. The long range PCR technique and/or screening of a mouse genomic library with probes derived from the 5'-end of the cDNAs can be used to isolate large exon/intron regions. We will then substitute one or more of the cloned genomic DNA exons for the neomycin resistance expression cassette for producing homologous recombination and knock-out mice. Alternatively, cDNAs coding for NLs will be used to overexpressed each of these enzymes in transgenic mice. The cDNAs will be cloned downstream from a promoter sequence, and injected in fertilised mouse eggs. Depending on specific questions to be answered, the chosen promoter sequence will allow expression of the peptidases either in every tissues or in a cell- or tissue-specific manner. Injected eggs will be transferred into foster mothers and the resulting mice analysed for peptidase expression;

replace defective NL genes in a gene therapy strategy. The NL full length cDNAs could be cloned under the control of a constitutive or inducible promoter having a narrow or wide range of tissue expression and introduced with appropriate vectors in subjects having defective genes;

synthesise oligonucleotides that could be used to interfere with the expression of the NLs. For example but not exclusively, oligonucleotides with antisens or ribozyme activity could be developed. These oligonucleotides could be introduced in subjects as described above;

isolate other members of the family. Screening cDNA and/or genomic libraries with these cDNA probes at low stringency may allow to clone new members of the NEP-like family. Alternatively, alignment of the sequences may allow one to design specific degenerate oligonucleotide primers for RT-PCR screening with mRNA from tissues such as but not exclusively, the hearth and the brain.

Production of recombinant NL-enzymes

As shown in the present work, recombinant active NL-enzymes can be obtained by expression of NL-cDNAs in mammalian cells. From past experience with neprilysin, another member of the family (Devault et al., 1988; Fossiez et al., 1992; Ellefsen et al., submitted), expression can also be performed in other expression systems after cloning of NL-cDNAs in appropriate expression vectors. These expression systems may include but not exclusively the baculovirus/insect cells or larvae system and the *Pichia pastoris*-based yeast system. Production of recombinant NL-enzymes includes the production of naturally occurring membrane bound or soluble forms of the proteins or genetically engineered soluble forms of the enzymes. The latter can be obtained by substituting the cytosolic and trans-membrane domain by a cleavable signal peptide such as that of proopiomelanocortin, but not exclusively, as done previously (Lemay et al., 1989) or by transforming by genetic manipulations the non-cleavable signal peptide membrane anchor domain into a cleavable signal peptide, as done previously (Lemire et al., 1997) or by fusion of the ectodomain of NL-enzymes to the amino-terminal domain (from the initiator methionine to amino acid residue 300) of naturally occurring soluble NLs such as, but not

exclusively, NL-1 as done in this work. These recombinant NLs could be used to:

find a substrate. A substrate can be identified using one of the following.

Screening of existing bioactive peptides. Peptides are incubated in the presence of NL-enzymes and subsequently analysed by HPLC for degradation. Degradation is observed by disappearance of the peak of substrate and the appearance of peaks of products;

Screening phage libraries specifically designed for the purpose (phage display library). Each phage expresses at its surface, as part of its coat protein, a random peptide sequence preceded by a peptide sequence recognisable by an antibody or any other sequence-recognizing agent. This latter sequence serves to attach the phage to a solid support. Upon addition of the NL-enzyme the random sequences that are NL substrate are cleaved, releasing the phage. After several rounds of cleavage, the phage sequence is determined to identify the peptide segment recognized by the enzyme.

Extract of the tissue where the enzyme is expressed is collected and prepared for chromatographic analysis (HPLC, capillary electrophoresis or any other high resolution separation system) by denaturing the extracted proteins with a solvent (acetonitrile or methanol). The extract is subjected to chromatographic separation. The same extract is incubated with the enzyme for a period sufficient to observe a difference between the 2 chromatograms. The regions with the identified changes are collected and subjected to mass spectrometric analysis to determine the peptide compositions.

Small peptide libraries are prepared with a fluorophore at one extremity and a quencher group at the other (Meldal et al Methods in molecular biology 1998, 87). The substrate can be identified using a strategy described in Apletalina et al (JBC (1998)273, 41, 26589-95). For each hexapeptide library, the identity of one residue at one position remains constant while the rest is randomized (for a total of $6 \times 20 = 120$ individual libraries). Each library is made-up of 3.2 million different members and is identified both by the position of the constant residue along the hexapeptide, and its identity. The NL-enzyme is added to each library and the fluorescence is recorded. The data is organized to identify the libraries producing the most fluorescence for each position along the hexapeptide. This arrangement suggests the identity of important residues at each position along the hexapeptide. Hexapeptide representing the best suggestions are prepared and tested in a similar fashion. From this set, the hexapeptide with the best fluorescence is selected.

set up enzymatic assays. An enzymatic assay consists in the addition of the above-identified substrate to the enzyme in constant conditions of pH, salts, temperature and time. The resulting solution is assayed for the hydrolysed peptide or for the intact peptide. This assay can be realized with specific antibodies, HPLC or, when self-quenched fluorescence tagged peptides are used (Meldal et al), by the appearance of fluorescence. The enzyme may be in solution or attached to a solid substrate;

identify inhibitors. Inhibitors can be identified from synthetic libraries, biota extracts and from rationally designed inhibitors using X-ray crystallography and

substituent activity relationships. Each molecule or extract fraction is tested for inhibitory activity using the enzymatic test described above. The molecule responsible for the largest inhibition is further tested to determine its pharmacological and toxicological properties following known procedures. The inhibitor with the best distribution, pharmacological action combined with low toxicity will be selected for drug manufacturing. Pharmaceutically acceptable formulation of the inhibitor or its acceptable salt will be prepared by mixing with known excipients to produce tablets, capsules or injectable solutions. Between 1 and 500 mg of the drug is administered to the patients;

inject the native or soluble purified NL-enzymes into subjects. In the case of disease or pathologies caused by a lack or decrease in NL activity, the purified NL could be injected intravenously or otherwise in patients. Alternatively, immobilized NL-enzymes could be introduced at the site of orthopedic surgery or implantation of devices in bones or dental tissues.

Secretion of foreign proteins and peptides

As shown in the present work, the amino-terminal domain of NL-1 (from the initiator methionine to the furin site) can be used to promote the secretion of a foreign protein (in this case the ectodomain of NL-3 and β -endorphin).

The amino-terminal domain of NL-1 but also of other naturally occurring soluble

NL-enzymes could be used to:

promote production and secretion of foreign proteins.

This can be achieved by genetically fusing sequences coding for said foreign proteins downstream from and in phase with the amino-terminal of NL-1. These chimeric constructs could be introduced with the help of appropriate vectors in any of the expression systems mentioned above for protein production and secretion;

promote production and secretion of bioactive peptides.

Sequences encoding small bioactive peptides such as but not exclusively β -endorphin, the enkephalins, substance P, atrial natriuretic peptide (ANF) and osteostatine, could be fused immediately downstream and in phase the furin site of NL-1. These DNA constructs could be used as described above to produce bioactive peptides.

serve as model to design artificial (non-naturally occurring) proteins or protein segments (protein vectors) to promote secretion of proteins or peptides. These protein vectors can be constructed to resemble a secreted protein. In this case they would be assembled of an endoplasmic reticulum signal peptide, a spacer of varying length and a furin cleavage site to which the protein or peptide destined for secretion can be fused. The total length of the spacer, furin cleavage site and protein or peptide destined for secretion must be at least 70 amino acid residues. Alternatively, such protein vectors could be assembled to resemble a type II membrane protein. In this case they would comprise from the amino to the carboxy-terminus a cytosolic domain of varying length, a transmembrane domain that also acts as a signal peptide, an extracellular segment of varying length and a furin cleavage site to which the protein or peptide destined for secretion can be fused. The total length of the extracellular segment, furin cleavage site and protein or peptide destined for secretion must be at least 70 amino acid residues.

Therapeutic applications of NL-enzymes

The inappropriate processing of endogenous peptides causes several diseases. The inappropriate processing may result from pathologic concentration of the enzyme itself, its substrate or other elements of the biochemical machinery downstream from the controlling enzyme. In this context it is possible to help the patient by managing the activity of the controlling enzyme.

NL-enzymes have been localized to the brain and may be involved in the improper processing of β -amyloid precursor. Inhibitions of this process by drugs prepared as above, will help patients with Alzheimer disease as well as other patient suffering from diseases caused by plaque formation;

NL-enzymes may be involved in the improper processing of other peptides involved in neurological diseases, pain or psychiatric disorders. Appropriately designed inhibitors will help in the management of such diseases;

NL-1 is found in testis and is associated with spermatozoid maturation. Peptides improperly processed by the enzyme may lead to infertility. The addition of NL-1 ex-vivo to seminal liquid or immature spermatozoids taken directly from testis during an in-vitro fertilization procedure will increase fertility. Conversely, the use of a small-molecule inhibitor or removal of NL-1 with an antibody could increase fertility during an in-vitro fertilization procedure. The administration of a NL-1 inhibitor may increase or decrease the fertility potential. This inhibitor is formulated and administered as described above.

NL-3 is found in ovaries and may be involved in the processing of a peptide involved in the maturation of eggs. The addition of NL-3 ex-vivo to immature eggs taken directly from ovaries during an in-vitro fertilization procedure will increase fertility. Conversely, the use of a small-molecule inhibitor or removal of NL-3 with an antibody could increase fertility during an in-vitro fertilization procedure. This inhibitor is formulated and administered as described above;

NL-3 is found in bones. The improper processing of peptides by the enzyme may result in bone disease or abnormal phosphate metabolism. Administration of an inhibitor, as described above, will allow the disease management.

TABLE I

	Extend of amino acid sequence identity between members of the NEP-like family								
	hNEP	hPEX	hECE-1A	hECE-2	hKELL	sNL-1	hNL-2	hNL-3	
hNEP	100*								
hPEX	35	100							
hECE-1A	39	38	100						
hECE-2	36	37	62	100					
hKELL	23	24	30	31	100				
sNL-1	55	39	39	39	26	100			
hNL-2	54	39	39	39	26	77	100		
hNL-3	35	32	37	37	28	36	34	100	

*percentage of sequence identity

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 675 680 685

Ala His Val Arg Cys Asn Ser Tyr Arg Pro Glu Ala Ala Arg Glu Gln
 690 695 700

Val Gln Ile Gly Ala His Ser Pro Pro Gln Phe Arg Val Asn Gly Ala
 705 710 715 720

Ile Ser Asn Phe Glu Glu Phe Gln Lys Ala Phe Asn Cys Pro Pro Asn
 725 730 735

Ser Thr Met Asn Arg Gly Met Asp Ser Cys Arg Leu Trp
 740 745

<210> SEQ ID NO 3
 <211> LENGTH: 732
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 3

Met Glu Gly Gly Asp Gln Ser Glu Glu Glu Pro Arg Glu Arg Ser Gln
 1 5 10 15

Ala Gly Gly Met Gly Thr Leu Trp Ser Gln Glu Ser Thr Pro Glu Glu
 20 25 30

Arg Leu Pro Val Glu Gly Ser Arg Pro Trp Ala Val Ala Arg Arg Val
 35 40 45

Leu Thr Ala Ile Leu Ile Leu Gly Leu Leu Leu Cys Phe Ser Val Leu
 50 55 60

Leu Phe Tyr Asn Phe Gln Asn Cys Gly Pro Arg Pro Cys Glu Thr Ser
 65 70 75 80

Val Cys Leu Asp Leu Arg Asp His Tyr Leu Ala Ser Gly Asn Thr Ser
 85 90 95

Val Ala Pro Cys Thr Asp Phe Phe Ser Phe Ala Cys Gly Arg Ala Lys
 100 105 110

Glu Thr Asn Asn Ser Phe Gln Glu Leu Ala Thr Lys Asn Lys Asn Arg
 115 120 125

Leu Arg Arg Ile Leu Glu Val Gln Asn Ser Trp His Pro Gly Ser Gly
 130 135 140

Glu Glu Lys Ala Phe Gln Phe Tyr Asn Ser Cys Met Asp Thr Leu Ala
 145 150 155 160

Ile Glu Ala Ala Gly Thr Gly Pro Leu Arg Gln Val Ile Glu Glu Leu
 165 170 175

Gly Gly Trp Arg Ile Ser Gly Lys Trp Thr Ser Leu Asn Phe Asn Arg
 180 185 190

Thr Leu Arg Leu Leu Met Ser Gln Tyr Gly His Phe Pro Phe Phe Arg
 195 200 205

Ala Tyr Leu Gly Pro His Pro Ala Ser Pro His Thr Pro Val Ile Gln
 210 215 220

Ile Asp Gln Pro Glu Phe Asp Val Pro Leu Lys Gln Asp Gln Glu Gln
 225 230 235 240

Lys Ile Tyr Ala Gln Ile Phe Arg Glu Tyr Leu Thr Tyr Leu Asn Gln
 245 250 255

Leu Gly Thr Leu Leu Gly Gly Asp Pro Ser Lys Val Gln Glu His Ser
 260 265 270

Ser Leu Ser Ile Ser Ile Thr Ser Arg Leu Phe Gln Phe Leu Arg Pro
 275 280 285

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Leu Glu Gln Arg Arg Ala Gln Gly Lys Leu Phe Gln Met Val Thr Ile
 290 295 300

Asp Gln Leu Lys Glu Met Ala Pro Ala Ile Asp Trp Leu Ser Cys Leu
 305 310 315 320

Gln Ala Thr Phe Thr Pro Met Ser Leu Ser Pro Ser Gln Ser Leu Val
 325 330 335

Val His Asp Val Glu Tyr Leu Lys Asn Met Ser Gln Leu Val Glu Glu
 340 345 350

Met Leu Leu Lys Gln Arg Asp Phe Leu Gln Ser His Met Ile Leu Gly
 355 360 365

Leu Val Val Thr Leu Ser Pro Ala Leu Asp Ser Gln Phe Gln Glu Ala
 370 375 380

Arg Arg Lys Leu Ser Gln Lys Leu Arg Glu Leu Thr Glu Gln Pro Pro
 385 390 395 400

Met Pro Ala Arg Pro Arg Trp Met Lys Cys Val Glu Glu Thr Gly Thr
 405 410 415

Phe Phe Glu Pro Thr Leu Ala Ala Leu Phe Val Arg Glu Ala Phe Gly
 420 425 430

Pro Ser Thr Arg Ser Ala Ala Met Lys Leu Phe Thr Ala Ile Arg Asp
 435 440 445

Ala Leu Ile Thr Arg Leu Arg Asn Leu Pro Trp Met Asn Glu Glu Thr
 450 455 460

Gln Asn Met Ala Gln Asp Lys Val Ala Gln Leu Gln Val Glu Met Gly
 465 470 475 480

Ala Ser Glu Trp Ala Leu Lys Pro Glu Leu Ala Arg Gln Glu Tyr Asn
 485 490 495

Asp Ile Gln Leu Gly Ser Ser Phe Leu Gln Ser Val Leu Ser Cys Val
 500 505 510

Arg Ser Leu Arg Ala Arg Ile Val Gln Ser Phe Leu Gln Pro His Pro
 515 520 525

Gln His Arg Trp Lys Val Ser Pro Trp Asp Val Asn Ala Tyr Tyr Ser
 530 535 540

Val Ser Asp His Val Val Val Phe Pro Ala Gly Leu Leu Gln Pro Pro
 545 550 555 560

Phe Phe His Pro Gly Tyr Pro Arg Ala Val Asn Phe Gly Ala Ala Gly
 565 570 575

Ser Ile Met Ala His Glu Leu Leu His Ile Phe Tyr Gln Leu Leu Leu
 580 585 590

Pro Gly Gly Cys Leu Ala Cys Asp Asn His Ala Leu Gln Glu Ala His
 595 600 605

Leu Cys Leu Lys Arg His Tyr Ala Ala Phe Pro Leu Pro Ser Arg Thr
 610 615 620

Ser Phe Asn Asp Ser Leu Thr Phe Leu Glu Asn Ala Ala Asp Val Gly
 625 630 635 640

Gly Leu Ala Ile Ala Leu Gln Ala Tyr Ser Lys Arg Leu Leu Arg His
 645 650 655

His Gly Glu Thr Val Leu Pro Ser Leu Asp Leu Ser Pro Gln Gln Ile
 660 665 670

Phe Phe Arg Ser Tyr Ala Gln Val Met Cys Arg Lys Pro Ser Pro Gln
 675 680 685

Asp Ser His Asp Thr His Ser Pro Pro His Leu Arg Val His Gly Pro
 690 695 700

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Leu Ser Ser Thr Pro Ala Phe Ala Arg Tyr Phe Arg Cys Ala Arg Gly
705 710 715 720

Ala Leu Leu Asn Pro Ser Ser Arg Cys Gln Leu Trp
725 730

<210> SEQ ID NO 4
<211> LENGTH: 753
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 4

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1 5 10 15

Ser Leu Ser Glu Gly Asp Ala Tyr Pro Asn Gly Leu Gln Val Asn Phe
20 25 30

His Ser Pro Arg Ser Gly Gln Arg Cys Trp Ala Ala Arg Thr Gln Val
35 40 45

Glu Lys Arg Leu Val Val Leu Val Val Leu Leu Ala Ala Gly Leu Val
50 55 60

Ala Cys Leu Ala Ala Leu Gly Ile Gln Tyr Gln Thr Arg Ser Pro Ser
65 70 75 80

Val Cys Leu Ser Glu Ala Cys Val Ser Val Thr Ser Ser Ile Leu Ser
85 90 95

Ser Met Asp Pro Thr Val Asp Pro Cys His Asp Phe Phe Ser Tyr Ala
100 105 110

Cys Gly Gly Trp Ile Lys Ala Asn Pro Val Pro Asp Gly His Ser Arg
115 120 125

Trp Gly Thr Phe Ser Asn Leu Trp Glu His Asn Gln Ala Ile Ile Lys
130 135 140

His Leu Leu Glu Asn Ser Thr Ala Ser Val Ser Glu Ala Glu Arg Lys
145 150 155 160

Ala Gln Val Tyr Tyr Arg Ala Cys Met Asn Glu Thr Arg Ile Glu Glu
165 170 175

Leu Arg Ala Lys Pro Leu Met Glu Leu Ile Glu Arg Leu Gly Gly Trp
180 185 190

Asn Ile Thr Gly Pro Trp Ala Lys Asp Asn Phe Gln Asp Thr Leu Gln
195 200 205

Val Val Thr Ala His Tyr Arg Thr Ser Pro Phe Phe Ser Val Tyr Val
210 215 220

Ser Ala Asp Ser Lys Asn Ser Asn Ser Asn Val Ile Gln Val Asp Gln
225 230 235 240

Ser Gly Leu Gly Leu Pro Ser Arg Asp Tyr Tyr Leu Asn Lys Thr Glu
245 250 255

Asn Glu Lys Val Leu Thr Gly Tyr Leu Asn Tyr Met Val Gln Leu Gly
260 265 270

Lys Leu Leu Gly Gly Gly Asp Glu Glu Ala Ile Arg Pro Gln Met Gln
275 280 285

Gln Ile Leu Asp Phe Glu Thr Ala Leu Ala Asn Ile Thr Ile Pro Gln
290 295 300

Glu Lys Arg Arg Asp Glu Glu Leu Ile Tyr His Lys Val Thr Ala Ala
305 310 315 320

Glu Leu Gln Thr Leu Ala Pro Ala Ile Asn Trp Leu Pro Phe Leu Asn
325 330 335

Thr Ile Phe Tyr Pro Val Glu Ile Asn Glu Ser Glu Pro Ile Val Val
340 345 350

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Tyr Asp Lys Glu Tyr Leu Glu Gln Ile Ser Thr Leu Ile Asn Thr Thr
 355 360 365
 Asp Arg Cys Leu Leu Asn Asn Tyr Met Ile Trp Asn Leu Val Arg Lys
 370 375 380
 Thr Ser Ser Phe Leu Asp Gln Arg Phe Gln Asp Ala Asp Glu Lys Phe
 385 390 395 400
 Met Glu Val Met Tyr Gly Thr Lys Lys Thr Cys Leu Pro Arg Trp Lys
 405 410 415
 Phe Cys Val Ser Asp Thr Glu Asn Asn Leu Gly Phe Ala Leu Gly Pro
 420 425 430
 Met Phe Val Lys Ala Thr Phe Ala Glu Asp Ser Lys Ser Ile Ala Thr
 435 440 445
 Glu Ile Ile Leu Glu Ile Lys Lys Ala Phe Glu Glu Ser Leu Ser Thr
 450 455 460
 Leu Lys Trp Met Asp Glu Glu Thr Arg Lys Ser Ala Lys Glu Lys Ala
 465 470 475 480
 Asp Ala Ile Tyr Asn Met Ile Gly Tyr Pro Asn Phe Ile Met Asp Pro
 485 490 495
 Lys Glu Leu Asp Lys Val Phe Asn Asp Tyr Thr Ala Val Pro Asp Leu
 500 505 510
 Tyr Phe Glu Asn Ala Met Arg Phe Phe Asn Phe Ser Trp Arg Val Thr
 515 520 525
 Ala Asp Gln Leu Arg Lys Ala Pro Asn Arg Asp Gln Trp Ser Met Thr
 530 535 540
 Pro Pro Met Val Asn Ala Tyr Tyr Ser Pro Thr Lys Asn Glu Ile Val
 545 550 555 560
 Phe Pro Ala Gly Ile Leu Gln Ala Pro Phe Tyr Thr Arg Ser Ser Pro
 565 570 575
 Lys Ala Leu Asn Phe Gly Gly Ile Gly Val Val Val Gly His Glu Leu
 580 585 590
 Thr His Ala Phe Asp Asp Gln Gly Arg Glu Tyr Asp Lys Asp Gly Asn
 595 600 605
 Leu Arg Pro Trp Trp Lys Asn Ser Ser Val Glu Ala Phe Lys Arg Gln
 610 615 620
 Thr Glu Cys Met Val Glu Gln Tyr Ser Asn Tyr Ser Val Asn Gly Glu
 625 630 635 640
 Pro Val Asn Gly Arg His Thr Leu Gly Glu Asn Ile Ala Asp Asn Gly
 645 650 655
 Gly Leu Lys Ala Ala Tyr Arg Ala Tyr Gln Asn Trp Val Lys Lys Asn
 660 665 670
 Gly Ala Glu His Ser Leu Pro Thr Leu Gly Leu Thr Asn Asn Gln Leu
 675 680 685
 Phe Phe Leu Gly Phe Ala Gln Val Trp Cys Ser Val Arg Thr Pro Glu
 690 695 700
 Ser Ser His Glu Gly Leu Ile Thr Asp Pro His Ser Pro Ser Arg Phe
 705 710 715 720
 Arg Val Ile Gly Ser Leu Ser Asn Ser Lys Glu Phe Ser Glu His Phe
 725 730 735
 Arg Cys Pro Pro Gly Ser Pro Met Asn Pro Pro His Lys Cys Glu Val
 740 745 750

Trp

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<210> SEQ ID NO 5
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
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<221> NAME/KEY: modified_base
<222> LOCATION: (14)
<223> OTHER INFORMATION: i
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<223> OTHER INFORMATION: i
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<221> NAME/KEY: modified_base
<222> LOCATION: (21)
<223> OTHER INFORMATION: i
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<223> OTHER INFORMATION: Description of Artificial Sequence:
      Oligonucleotide primers for RT-PCR reactions

<400> SEQUENCE: 5

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tggatggatc gacngganac naca

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24

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<210> SEQ ID NO 6
<211> LENGTH: 24
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<213> ORGANISM: Artificial Sequence
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<222> LOCATION: (14)
<223> OTHER INFORMATION: i
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<221> NAME/KEY: modified_base
<222> LOCATION: (21)
<223> OTHER INFORMATION: i
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<400> SEQUENCE: 6

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24

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<210> SEQ ID NO 7
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<223> OTHER INFORMATION: i
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<223> OTHER INFORMATION: i
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<223> OTHER INFORMATION: Description of Artificial Sequence:
Oligonucleotide primers for RT-PCR reactions

<400> SEQUENCE: 7

agtngtnttt cccngcnggn agtancttat ca

32

<210> SEQ ID NO 8
 <211> LENGTH: 32
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
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 <222> LOCATION: (4)
 <223> OTHER INFORMATION: i
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Oligonucleotide primers for RT-PCR reactions

<400> SEQUENCE: 8

agtngtnttt cccngcnggn agtancttgc ca

32

<210> SEQ ID NO 9
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 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
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 <222> LOCATION: (2)
 <223> OTHER INFORMATION: i
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 <222> LOCATION: (18)
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 <220> FEATURE:
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Oligonucleotide primers for RT-PCR reactions
 <220> FEATURE:
 <221> NAME/KEY: modified_base
 <222> LOCATION: (3)
 <223> OTHER INFORMATION: i

<400> SEQUENCE: 9

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annccnccna tctagtcngc ngactagttt ctc

33

<210> SEQ ID NO 10
 <211> LENGTH: 35
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
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 <222> LOCATION: (12)
 <223> OTHER INFORMATION: i
 <220> FEATURE:
 <221> NAME/KEY: modified_base
 <222> LOCATION: (24)
 <223> OTHER INFORMATION: i
 <220> FEATURE:
 <223> OTHER INFORMATION: Description of Artificial Sequence:
 Oligonucleotide primers for RT-PCR reactions

<400> SEQUENCE: 10

gatcaatctc tngatcgaag tctnaatctg gatgg

35

<210> SEQ ID NO 11
 <211> LENGTH: 37
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
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 <222> LOCATION: (9)
 <223> OTHER INFORMATION: i
 <220> FEATURE:
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 <223> OTHER INFORMATION: i
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 Oligonucleotide primers for RT-PCR reactions

<400> SEQUENCE: 11

tctcaccana tntgagcat cgttcttcat ngggatg

37

<210> SEQ ID NO 12
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 <212> TYPE: DNA
 <213> ORGANISM: Mus musculus
 <220> FEATURE:
 <221> NAME/KEY: CDS
 <222> LOCATION: (332)..(2626)

<400> SEQUENCE: 12

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accttggcca gctcacccca actctgagac atcccaacct agcctttaag gacttgccca 120

gcagtgactg agagcaccag ggtcccctgg gcacttgggg cacagcttac agcattgaga 180

gcagagacca ggacagtgca ccagcttcag tgtgtcctag gcatccgatc cgggctccag 240

ctgcctctct cctagccctg gcctgggggg cttagcgggtg tgccttccac ccagaaccgg 300

ctgatagggg aagtctgaga gcccagtggg g atg gtg gag aga gca ggc tgg 352
 Met Val Glu Arg Ala Gly Trp
 1 5

tgt cgg aag aag tcc cca ggc ttc gtg gag tat ggg ctg atg gtg ctg 400
 Cys Arg Lys Lys Ser Pro Gly Phe Val Glu Tyr Gly Leu Met Val Leu
 10 15 20

ctg ctg ctg ttg ctg gga gcc ata gtg act ctg ggt gtc ttc tac agc 448
 Leu Leu Leu Leu Leu Gly Ala Ile Val Thr Leu Gly Val Phe Tyr Ser

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25	30	35	
ata ggg aag cag ctg ccc ctc tta act agc ctg cta cac ttc tcc tgg Ile Gly Lys Gln Leu Pro Leu Leu Thr Ser Leu Leu His Phe Ser Trp 40 45 50 55			496
gat gag agg acg gtt gta aaa cga gcc ctc agg gat tca tca ctg aaa Asp Glu Arg Thr Val Val Lys Arg Ala Leu Arg Asp Ser Ser Leu Lys 60 65 70			544
agt gac atc tgc acc acc cca agc tgt gtg ata gca gct gcc aga atc Ser Asp Ile Cys Thr Thr Pro Ser Cys Val Ile Ala Ala Ala Arg Ile 75 80 85			592
ctc gaa aac atg gac caa tcg agg aac ccc tgt gaa aac ttc tac cag Leu Glu Asn Met Asp Gln Ser Arg Asn Pro Cys Glu Asn Phe Tyr Gln 90 95 100			640
tac gcc tgc gga ggc tgg ctg agg cac cac gtg atc cca gag acc aac Tyr Ala Cys Gly Gly Trp Leu Arg His His Val Ile Pro Glu Thr Asn 105 110 115			688
tcc cga tac agc gtc ttt gac atc ctg cgg gac gag ctg gag gtt atc Ser Arg Tyr Ser Val Phe Asp Ile Leu Arg Asp Glu Leu Glu Val Ile 120 125 130 135			736
ctc aaa ggg gtg ctg gag gat tcc act tcc cag cat cgc ccg gcc gtg Leu Lys Gly Val Leu Glu Asp Ser Thr Ser Gln His Arg Pro Ala Val 140 145 150			784
gag aag gcc aag aca cta tat cgc tcc tgc atg aac caa agt gtg atc Glu Lys Ala Lys Thr Leu Tyr Arg Ser Cys Met Asn Gln Ser Val Ile 155 160 165			832
gag aag aga gac tct gag ccc ctg ctg agc gtc tta aaa atg gta gga Glu Lys Arg Asp Ser Glu Pro Leu Leu Ser Val Leu Lys Met Val Gly 170 175 180			880
ggt tgg cct gtg gcc atg gat aag tgg aac gag acc atg ggc ctc aag Gly Trp Pro Val Ala Met Asp Lys Trp Asn Glu Thr Met Gly Leu Lys 185 190 195			928
tgg gaa ctg gag cga cag ttg gct gtg ttg aac tcg cag ttc aac agg Trp Glu Leu Glu Arg Gln Leu Ala Val Leu Asn Ser Gln Phe Asn Arg 200 205 210 215			976
cgg gtc ctc atc gac ctc ttc atc tgg aat gac gac cag aac tcc agc Arg Val Leu Ile Asp Leu Phe Ile Trp Asn Asp Asp Gln Asn Ser Ser 220 225 230			1024
cgg cat gtc atc tac ata gac cag ccc acc ttg ggc atg cca tcc cgg Arg His Val Ile Tyr Ile Asp Gln Pro Thr Leu Gly Met Pro Ser Arg 235 240 245			1072
gag tac tat ttc cag gag gac aac aac cac aag gta cgg aaa gcc tac Glu Tyr Tyr Phe Gln Glu Asp Asn Asn His Lys Val Arg Lys Ala Tyr 250 255 260			1120
ctg gag ttc atg acg tca gtg gcc act atg ctt agg aaa gac cag aac Leu Glu Phe Met Thr Ser Val Ala Thr Met Leu Arg Lys Asp Gln Asn 265 270 275			1168
ctg tcc aag gag agc gcc atg gtg cgg gag gag atg gcg gag gtg ctg Leu Ser Lys Glu Ser Ala Met Val Arg Glu Glu Met Ala Glu Val Leu 280 285 290 295			1216
gaa ctg gag acg cat ctg gcc aac gcc aca gtc ccc cag gag aaa agg Glu Leu Glu Thr His Leu Ala Asn Ala Thr Val Pro Gln Glu Lys Arg 300 305 310			1264
cat gat gtc act gcc ctg tac cac cga atg gac ctg atg gag cta cag His Asp Val Thr Ala Leu Tyr His Arg Met Asp Leu Met Glu Leu Gln 315 320 325			1312
gaa agg ttt ggt ctg aag ggg ttt aac tgg act ctc ttc ata caa aac Glu Arg Phe Gly Leu Lys Gly Phe Asn Trp Thr Leu Phe Ile Gln Asn 330 335 340			1360
gtg ttg tct tct gtg gaa gtc gag ctg ttc cca gat gag gag gtg gtg			1408

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Val 345	Leu	Ser	Ser	Val	Glu 350	Val	Glu	Leu	Phe	Pro	Asp 355	Glu	Glu	Val	Val	
gtc	tac	ggc	atc	ccc	tac	ctg	gag	aat	ctg	gag	gat	atc	att	gat	agc	1456
Val 360	Tyr	Gly	Ile	Pro	Tyr 365	Leu	Glu	Asn	Leu	Glu	Asp 370	Ile	Ile	Asp	Ser 375	
tac	tca	gca	cgg	acc	atg	cag	aac	tac	ctg	gta	tgg	cgc	ctg	gtg	cta	1504
Tyr	Ser	Ala	Arg	Thr 380	Met	Gln	Asn	Tyr	Leu	Val	Trp	Arg	Leu	Val	Leu 390	
gat	cga	att	ggc	agc	ctg	agc	cag	aga	ttc	aaa	gag	gcg	cgt	gtg	gac	1552
Asp	Arg	Ile	Gly	Ser 395	Leu	Ser	Gln	Arg	Phe 400	Lys	Glu	Ala	Arg	Val	Asp 405	
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Tyr	Arg	Lys	Ala	Leu 410	Tyr	Gly	Thr	Thr	Val	Glu	Glu	Val	Arg	Trp	Arg 420	
gag	tgt	gtc	agc	tat	gtc	aac	agt	aac	atg	gag	agc	gcc	gtg	ggc	tcc	1648
Glu	Cys	Val	Ser	Tyr 425	Val	Asn	Ser	Asn	Met	Glu	Ser	Ala	Val	Gly	Ser 435	
ctc	tac	atc	aag	cgg	gcc	ttc	tcc	aag	gac	agc	aag	agc	acg	gtc	aga	1696
Leu	Tyr	Ile	Lys	Arg 440	Ala	Phe	Ser	Lys	Asp	Ser	Lys	Ser	Thr	Val	Arg 455	
gag	ctg	att	gag	aag	ata	agg	tcc	gtg	ttt	gtg	gat	aac	ctg	gat	gag	1744
Glu	Leu	Ile	Glu	Lys 460	Ile	Arg	Ser	Val	Phe	Val	Asp	Asn	Leu	Asp	Glu 470	
ctg	aac	tgg	atg	gac	gag	gaa	tcc	aag	aag	aag	gcc	cag	gaa	aag	gcc	1792
Leu	Asn	Trp	Met	Asp 475	Glu	Glu	Ser	Lys	Lys	Lys	Ala	Gln	Glu	Lys	Ala 485	
atg	aat	ata	cgg	gaa	cag	att	ggc	tac	cct	gac	tac	att	ttg	gaa	gat	1840
Met	Asn	Ile	Arg	Glu 490	Gln	Ile	Gly	Tyr	Pro	Asp	Tyr	Ile	Leu	Glu	Asp 500	
aac	aat	aaa	cac	ctg	gat	gag	gaa	tac	tcc	agt	ttg	act	ttc	tat	gag	1888
Asn	Asn	Lys	His	Leu 505	Asp	Glu	Glu	Tyr	Ser	Ser	Leu	Thr	Phe	Tyr	Glu 515	
gac	ctg	tat	ttt	gag	aac	gga	ctt	cag	aac	ctc	aag	aac	aat	gcc	cag	1936
Asp	Leu	Tyr	Phe	Glu 520	Asn	Gly	Leu	Gln	Asn	Leu	Lys	Asn	Asn	Ala	Gln 535	
agg	agc	ctc	aag	aag	ctt	cgg	gaa	aag	gtg	gac	cag	aat	ctc	tgg	atc	1984
Arg	Ser	Leu	Lys	Lys 540	Leu	Arg	Glu	Lys	Val	Asp	Gln	Asn	Leu	Trp	Ile 550	
atc	ggg	gct	gca	gtg	gtc	aat	gca	ttc	tac	tcc	cca	aac	aga	aac	cag	2032
Ile	Gly	Ala	Ala	Val 555	Val	Asn	Ala	Phe	Tyr	Ser	Pro	Asn	Arg	Asn	Gln 565	
atc	gtc	ttt	cca	gca	ggg	att	ctc	cag	ccg	ccc	ttc	ttc	agc	aag	gac	2080
Ile	Val	Phe	Pro	Ala 570	Gly	Ile	Leu	Gln	Pro	Pro	Phe	Phe	Ser	Lys	Asp 580	
caa	cca	cag	tcc	ttg	aat	ttt	ggg	ggc	atc	ggg	atg	gtg	att	ggg	cac	2128
Gln	Pro	Gln	Ser	Leu 585	Asn	Phe	Gly	Gly	Ile	Gly	Met	Val	Ile	Gly	His 595	
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Glu	Ile	Thr	His	Gly 600	Phe	Asp	Asp	Asn	Gly	Arg	Asn	Phe	Asp	Lys	Asn 615	
ggc	aac	atg	ctg	gac	tgg	tgg	agt	aac	ttc	tcg	gcc	cgg	cac	ttc	caa	2224
Gly	Asn	Met	Leu	Asp 620	Trp	Trp	Ser	Asn	Phe	Ser	Ala	Arg	His	Phe	Gln 630	
cag	cag	tcg	caa	tgc	atg	atc	tat	cag	tac	ggc	aac	ttc	tct	tgg	gaa	2272
Gln	Gln	Ser	Gln	Cys 635	Met	Ile	Tyr	Gln	Tyr	Gly	Asn	Phe	Ser	Trp	Glu 645	
cta	gca	gac	aac	cag	aat	gtg	aac	gga	ttc	agt	acc	ctc	ggg	gag	aac	2320
Leu	Ala	Asp	Asn	Gln 650	Asn	Val	Asn	Gly	Phe	Ser	Thr	Leu	Gly	Glu	Asn 660	

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att gcc gac aac gga ggt gtg cga cag gca tac aag gct tac cta cgg	2368
Ile Ala Asp Asn Gly Gly Val Arg Gln Ala Tyr Lys Ala Tyr Leu Arg	
665 670 675	
tgg ctg gct gat ggc ggc aaa gat cag cga ctg ccg gga ctg aac ctg	2416
Trp Leu Ala Asp Gly Gly Lys Asp Gln Arg Leu Pro Gly Leu Asn Leu	
680 685 690 695	
acc tat gcc cag ctt ttc ttc atc aac tat gcc cag gtg tgg tgt ggg	2464
Thr Tyr Ala Gln Leu Phe Phe Ile Asn Tyr Ala Gln Val Trp Cys Gly	
700 705 710	
tcc tat agg ccg gag ttc gcc gtc cag tcc atc aag acg gac gtc cac	2512
Ser Tyr Arg Pro Glu Phe Ala Val Gln Ser Ile Lys Thr Asp Val His	
715 720 725	
agt cct ctt aag tac agg gtg ctg ggc tca cta cag aac ctg cca ggc	2560
Ser Pro Leu Lys Tyr Arg Val Leu Gly Ser Leu Gln Asn Leu Pro Gly	
730 735 740	
ttc tct gag gca ttc cac tgc cca cga ggc agc ccc atg cac ccc atg	2608
Phe Ser Glu Ala Phe His Cys Pro Arg Gly Ser Pro Met His Pro Met	
745 750 755	
aag cga tgt cgc atc tgg tagccaaggc tgagctatgc tgcggcccac	2656
Lys Arg Cys Arg Ile Trp	
760 765	
gccccgccac ccagaggctt cgcgaatggt gtagctggca gagatgtgca ggtctttgcc	2716
tgaaggccac cggagccacc agccagccct ccgcgccag cctagagtgt agccaccgc	2776
ccacacccgg gatgagtgtt gccggtcctg cgcctcctca ggccagtgtg ggtcagcagc	2836
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gcttcagact tgagctaagt aaacgcttc	2925

<210> SEQ ID NO 13

<211> LENGTH: 765

<212> TYPE: PRT

<213> ORGANISM: Mus musculus

<400> SEQUENCE: 13

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20 25 30	
Thr Leu Gly Val Phe Tyr Ser Ile Gly Lys Gln Leu Pro Leu Leu Thr	
35 40 45	
Ser Leu Leu His Phe Ser Trp Asp Glu Arg Thr Val Val Lys Arg Ala	
50 55 60	
Leu Arg Asp Ser Ser Leu Lys Ser Asp Ile Cys Thr Thr Pro Ser Cys	
65 70 75 80	
Val Ile Ala Ala Ala Arg Ile Leu Glu Asn Met Asp Gln Ser Arg Asn	
85 90 95	
Pro Cys Glu Asn Phe Tyr Gln Tyr Ala Cys Gly Gly Trp Leu Arg His	
100 105 110	
His Val Ile Pro Glu Thr Asn Ser Arg Tyr Ser Val Phe Asp Ile Leu	
115 120 125	
Arg Asp Glu Leu Glu Val Ile Leu Lys Gly Val Leu Glu Asp Ser Thr	
130 135 140	
Ser Gln His Arg Pro Ala Val Glu Lys Ala Lys Thr Leu Tyr Arg Ser	
145 150 155 160	
Cys Met Asn Gln Ser Val Ile Glu Lys Arg Asp Ser Glu Pro Leu Leu	
165 170 175	

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Ser	Val	Leu	Lys	Met	Val	Gly	Gly	Trp	Pro	Val	Ala	Met	Asp	Lys	Trp
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Asn	Glu	Thr	Met	Gly	Leu	Lys	Trp	Glu	Leu	Glu	Arg	Gln	Leu	Ala	Val
		195					200					205			
Leu	Asn	Ser	Gln	Phe	Asn	Arg	Arg	Val	Leu	Ile	Asp	Leu	Phe	Ile	Trp
	210					215					220				
Asn	Asp	Asp	Gln	Asn	Ser	Ser	Arg	His	Val	Ile	Tyr	Ile	Asp	Gln	Pro
225					230					235					240
Thr	Leu	Gly	Met	Pro	Ser	Arg	Glu	Tyr	Tyr	Phe	Gln	Glu	Asp	Asn	Asn
				245					250					255	
His	Lys	Val	Arg	Lys	Ala	Tyr	Leu	Glu	Phe	Met	Thr	Ser	Val	Ala	Thr
			260					265					270		
Met	Leu	Arg	Lys	Asp	Gln	Asn	Leu	Ser	Lys	Glu	Ser	Ala	Met	Val	Arg
		275					280					285			
Glu	Glu	Met	Ala	Glu	Val	Leu	Glu	Leu	Glu	Thr	His	Leu	Ala	Asn	Ala
	290					295					300				
Thr	Val	Pro	Gln	Glu	Lys	Arg	His	Asp	Val	Thr	Ala	Leu	Tyr	His	Arg
305					310					315					320
Met	Asp	Leu	Met	Glu	Leu	Gln	Glu	Arg	Phe	Gly	Leu	Lys	Gly	Phe	Asn
				325					330					335	
Trp	Thr	Leu	Phe	Ile	Gln	Asn	Val	Leu	Ser	Ser	Val	Glu	Val	Glu	Leu
			340					345					350		
Phe	Pro	Asp	Glu	Glu	Val	Val	Val	Tyr	Gly	Ile	Pro	Tyr	Leu	Glu	Asn
		355					360					365			
Leu	Glu	Asp	Ile	Ile	Asp	Ser	Tyr	Ser	Ala	Arg	Thr	Met	Gln	Asn	Tyr
	370					375					380				
Leu	Val	Trp	Arg	Leu	Val	Leu	Asp	Arg	Ile	Gly	Ser	Leu	Ser	Gln	Arg
385					390					395					400
Phe	Lys	Glu	Ala	Arg	Val	Asp	Tyr	Arg	Lys	Ala	Leu	Tyr	Gly	Thr	Thr
				405					410					415	
Val	Glu	Glu	Val	Arg	Trp	Arg	Glu	Cys	Val	Ser	Tyr	Val	Asn	Ser	Asn
			420					425					430		
Met	Glu	Ser	Ala	Val	Gly	Ser	Leu	Tyr	Ile	Lys	Arg	Ala	Phe	Ser	Lys
		435					440					445			
Asp	Ser	Lys	Ser	Thr	Val	Arg	Glu	Leu	Ile	Glu	Lys	Ile	Arg	Ser	Val
	450					455					460				
Phe	Val	Asp	Asn	Leu	Asp	Glu	Leu	Asn	Trp	Met	Asp	Glu	Glu	Ser	Lys
465					470					475					480
Lys	Lys	Ala	Gln	Glu	Lys	Ala	Met	Asn	Ile	Arg	Glu	Gln	Ile	Gly	Tyr
				485					490					495	
Pro	Asp	Tyr	Ile	Leu	Glu	Asp	Asn	Asn	Lys	His	Leu	Asp	Glu	Glu	Tyr
			500					505					510		
Ser	Ser	Leu	Thr	Phe	Tyr	Glu	Asp	Leu	Tyr	Phe	Glu	Asn	Gly	Leu	Gln
		515					520					525			
Asn	Leu	Lys	Asn	Asn	Ala	Gln	Arg	Ser	Leu	Lys	Lys	Leu	Arg	Glu	Lys
	530					535					540				
Val	Asp	Gln	Asn	Leu	Trp	Ile	Ile	Gly	Ala	Ala	Val	Val	Asn	Ala	Phe
545					550					555					560
Tyr	Ser	Pro	Asn	Arg	Asn	Gln	Ile	Val	Phe	Pro	Ala	Gly	Ile	Leu	Gln
				565					570					575	
Pro	Pro	Phe	Phe	Ser	Lys	Asp	Gln	Pro	Gln	Ser	Leu	Asn	Phe	Gly	Gly
			580					585					590		
Ile	Gly	Met	Val	Ile	Gly	His	Glu	Ile	Thr	His	Gly	Phe	Asp	Asp	Asn

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595					600					605						
Gly	Arg	Asn	Phe	Asp	Lys	Asn	Gly	Asn	Met	Leu	Asp	Trp	Trp	Ser	Asn	
610					615					620						
Phe	Ser	Ala	Arg	His	Phe	Gln	Gln	Gln	Ser	Gln	Cys	Met	Ile	Tyr	Gln	
625					630					635					640	
Tyr	Gly	Asn	Phe	Ser	Trp	Glu	Leu	Ala	Asp	Asn	Gln	Asn	Val	Asn	Gly	
				645					650					655		
Phe	Ser	Thr	Leu	Gly	Glu	Asn	Ile	Ala	Asp	Asn	Gly	Gly	Val	Arg	Gln	
			660					665					670			
Ala	Tyr	Lys	Ala	Tyr	Leu	Arg	Trp	Leu	Ala	Asp	Gly	Gly	Lys	Asp	Gln	
		675					680						685			
Arg	Leu	Pro	Gly	Leu	Asn	Leu	Thr	Tyr	Ala	Gln	Leu	Phe	Phe	Ile	Asn	
	690					695					700					
Tyr	Ala	Gln	Val	Trp	Cys	Gly	Ser	Tyr	Arg	Pro	Glu	Phe	Ala	Val	Gln	
705					710					715					720	
Ser	Ile	Lys	Thr	Asp	Val	His	Ser	Pro	Leu	Lys	Tyr	Arg	Val	Leu	Gly	
				725					730					735		
Ser	Leu	Gln	Asn	Leu	Pro	Gly	Phe	Ser	Glu	Ala	Phe	His	Cys	Pro	Arg	
			740					745					750			
Gly	Ser	Pro	Met	His	Pro	Met	Lys	Arg	Cys	Arg	Ile	Trp				
		755					760					765				
<210> SEQ ID NO 14 <211> LENGTH: 2676 <212> TYPE: DNA <213> ORGANISM: Homo sapiens <220> FEATURE: <221> NAME/KEY: CDS <222> LOCATION: (7)..(2316) <400> SEQUENCE: 14																
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	Met	Val	Glu	Ser	Ala	Gly	Arg	Ala	Gly	Gln	Lys	Arg	Pro	Gly		
	1				5					10						
ttc	ctg	gag	ggg	ggg	ctg	ctg	ctg	ctg	ctg	ctg	gtg	acc	gct	gcc	96	
Phe	Leu	Glu	Gly	Gly	Leu	Leu	Leu	Leu	Leu	Leu	Val	Thr	Ala	Ala		
15					20					25				30		
ctg	gtg	gcc	ttg	ggt	gtc	ctc	tac	gcc	gac	cgc	aga	ggg	aag	cag	ctg	144
Leu	Val	Ala	Leu	Gly	Val	Leu	Tyr	Ala	Asp	Arg	Arg	Gly	Lys	Gln	Leu	
				35				40						45		
cca	cgc	ctt	gct	agc	cgg	ctg	tgc	ttc	tta	cag	gag	gag	agg	acc	ttt	192
Pro	Arg	Leu	Ala	Ser	Arg	Leu	Cys	Phe	Leu	Gln	Glu	Glu	Arg	Thr	Phe	
		50						55						60		
gta	aaa	cga	aaa	ccc	cga	ggg	atc	cca	gag	gcc	caa	gag	gtg	agc	gag	240
Val	Lys	Arg	Lys	Pro	Arg	Gly	Ile	Pro	Glu	Ala	Gln	Glu	Val	Ser	Glu	
		65					70						75			
gtc	tgc	acc	acc	cct	ggc	tgc	gtg	ata	gca	gcc	gcc	agg	atc	ctc	cag	288
Val	Cys	Thr	Thr	Pro	Gly	Cys	Val	Ile	Ala	Ala	Ala	Arg	Ile	Leu	Gln	
	80					85					90					
aac	atg	gac	ccg	acc	acg	gaa	ccg	tgt	gac	gac	ttc	tac	cag	ttt	gca	336
Asn	Met	Asp	Pro	Thr	Thr	Glu	Pro	Cys	Asp	Asp	Phe	Tyr	Gln	Phe	Ala	
	95				100					105					110	
tgc	gga	ggc	tgg	ctg	cgg	cgc	cac	gtg	atc	cct	gag	acc	aac	tca	aga	384
Cys	Gly	Gly	Trp	Leu	Arg	Arg	His	Val	Ile	Pro	Glu	Thr	Asn	Ser	Arg	
				115					120					125		
tac	agc	atc	ttt	gac	gtc	ctc	cgc	gac	gag	ctg	gag	gtc	atc	ctc	aaa	432
Tyr	Ser	Ile	Phe	Asp	Val	Leu	Arg	Asp	Glu	Leu	Glu	Val	Ile	Leu	Lys	
			130					135						140		

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gcg gtg ctg gag aat tcg act gcc aag gac cgg ccg gct gtg gag aag	480
Ala Val Leu Glu Asn Ser Thr Ala Lys Asp Arg Pro Ala Val Glu Lys	
145 150 155	
gcc agg acg ctg tac cgc tcc tgc atg aac cag agt gtg ata gag aag	528
Ala Arg Thr Leu Tyr Arg Ser Cys Met Asn Gln Ser Val Ile Glu Lys	
160 165 170	
cga ggc tct cag ccc ctg ctg gac atc ttg gag gtg gtg gga ggc tgg	576
Arg Gly Ser Gln Pro Leu Leu Asp Ile Leu Glu Val Val Gly Gly Trp	
175 180 185 190	
ccg gtg gcg atg gac agg tgg aac gag acc gta gga ctc gag tgg gag	624
Pro Val Ala Met Asp Arg Trp Asn Glu Thr Val Gly Leu Glu Trp Glu	
195 200 205	
ctg gag cgg cag ctg gcg ctg atg aac tca cag ttc aac agg cgc gtc	672
Leu Glu Arg Gln Leu Ala Leu Met Asn Ser Gln Phe Asn Arg Arg Val	
210 215 220	
ctc atc gac ctc ttc atc tgg aac gac gac cag aac tcc agc cgg cac	720
Leu Ile Asp Leu Phe Ile Trp Asn Asp Asp Gln Asn Ser Ser Arg His	
225 230 235	
atc atc tac ata gac cag ccc acc ttg ggc atg ccc tcc cga gag tac	768
Ile Ile Tyr Ile Asp Gln Pro Thr Leu Gly Met Pro Ser Arg Glu Tyr	
240 245 250	
tac ttc aac ggc ggc agc aac cgg aag gtg cgg gaa gcc tac ctg cag	816
Tyr Phe Asn Gly Gly Ser Asn Arg Lys Val Arg Glu Ala Tyr Leu Gln	
255 260 265 270	
ttc atg gtg tca gtg gcc acg ttg ctg cgg gag gat gca aac ctg ccc	864
Phe Met Val Ser Val Ala Thr Leu Leu Arg Glu Asp Ala Asn Leu Pro	
275 280 285	
agg gac agc tgc ctg gtg cag gag gac atg gtg cag gtt ctg gag ctg	912
Arg Asp Ser Cys Leu Val Gln Glu Asp Met Val Gln Val Leu Glu Leu	
290 295 300	
gag aca cag ctg gcc aag gcc acg gta ccc cag gag gag aga cac gac	960
Glu Thr Gln Leu Ala Lys Ala Thr Val Pro Gln Glu Glu Arg His Asp	
305 310 315	
gtc atc gcc ttg tac cac cgg atg gga ctg gag gag ctg caa agc cag	1008
Val Ile Ala Leu Tyr His Arg Met Gly Leu Glu Glu Leu Gln Ser Gln	
320 325 330	
ttt ggc ctg aag gga ttt aac tgg act ctg ttc ata caa act gtg cta	1056
Phe Gly Leu Lys Gly Phe Asn Trp Thr Leu Phe Ile Gln Thr Val Leu	
335 340 345 350	
tcc tct gtc aaa atc aag ctg ctg cca gat gag gaa gtg gtg gtc tat	1104
Ser Ser Val Lys Ile Lys Leu Leu Pro Asp Glu Glu Val Val Val Tyr	
355 360 365	
ggc atc ccc tac ctg cag aac ctt gaa aac atc atc gac acc tac tca	1152
Gly Ile Pro Tyr Leu Gln Asn Leu Glu Asn Ile Ile Asp Thr Tyr Ser	
370 375 380	
gcc agg acc ata cag aac tac ctg gtc tgg cgc ctg gtg ctg gac cgc	1200
Ala Arg Thr Ile Gln Asn Tyr Leu Val Trp Arg Leu Val Leu Asp Arg	
385 390 395	
att ggt agc cta agc cag aga ttc aag gac aca cga gtg aac tac cgc	1248
Ile Gly Ser Leu Ser Gln Arg Phe Lys Asp Thr Arg Val Asn Tyr Arg	
400 405 410	
aag gcg ctg ttt ggc aca atg gtg gag gag gtg cgc tgg cgt gaa tgt	1296
Lys Ala Leu Phe Gly Thr Met Val Glu Glu Val Arg Trp Arg Glu Cys	
415 420 425 430	
gtg ggc tac gtc aac agc aac atg gag aac gcc gtg ggc tcc ctc tac	1344
Val Gly Tyr Val Asn Ser Asn Met Glu Asn Ala Val Gly Ser Leu Tyr	
435 440 445	
gtc agg gag gcg ttc cct gga gac agc aag agc atg gtc aga gaa ctc	1392
Val Arg Glu Ala Phe Pro Gly Asp Ser Lys Ser Met Val Arg Glu Leu	
450 455 460	

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tgg atg gac gag gag tcc aag aag aag gcg cag gag aag gcc atg agc Trp Met Asp Glu Glu Ser Lys Lys Lys Ala Gln Glu Lys Ala Met Ser 480 485 490	1488
atc cgg gag cag atc ggg cac cct gac tac atc ctg gag gag atg aac Ile Arg Glu Gln Ile Gly His Pro Asp Tyr Ile Leu Glu Glu Met Asn 495 500 505 510	1536
agg cgc ctg gac gag gag tac tcc aat ctg aac ttc tca gag gac ctg Arg Arg Leu Asp Glu Glu Tyr Ser Asn Leu Asn Phe Ser Glu Asp Leu 515 520 525	1584
tac ttt gag aac agt ctg cag aac ctc aag gtg ggc gcc cag cgg agc Tyr Phe Glu Asn Ser Leu Gln Asn Leu Lys Val Gly Ala Gln Arg Ser 530 535 540	1632
ctc agg aag ctt cgg gaa aag gtg gac cca aat ctc tgg atc atc ggg Leu Arg Lys Leu Arg Glu Lys Val Asp Pro Asn Leu Trp Ile Ile Gly 545 550 555	1680
gcg gcg gtg gtc aat gcg ttc tac tcc cca aac cga aac cag att gta Ala Ala Val Val Asn Ala Phe Tyr Ser Pro Asn Arg Asn Gln Ile Val 560 565 570	1728
ttc cct gcc ggg atc ctc cag ccc ccc ttc ttc agc aag gag cag cca Phe Pro Ala Gly Ile Leu Gln Pro Pro Phe Phe Ser Lys Glu Gln Pro 575 580 585 590	1776
cag gcc ttg aac ttt gga ggc att ggg atg gtg atc ggg cac gag atc Gln Ala Leu Asn Phe Gly Gly Ile Gly Met Val Ile Gly His Glu Ile 595 600 605	1824
acg cac ggc ttt gac gac aat ggc cgg aac ttc gac aag aat ggc aac Thr His Gly Phe Asp Asp Asn Gly Arg Asn Phe Asp Lys Asn Gly Asn 610 615 620	1872
atg atg gat tgg tgg agt aac ttc tcc acc cag cac ttc cgg gag cag Met Met Asp Trp Trp Ser Asn Phe Ser Thr Gln His Phe Arg Glu Gln 625 630 635	1920
tca gag tgc atg atc tac cag tac gcc aac tac tcc tgg gac ctg gca Ser Glu Cys Met Ile Tyr Gln Tyr Gly Asn Tyr Ser Trp Asp Leu Ala 640 645 650	1968
gac gaa cag aac gtg aac gga ttc aac acc ctt ggg gaa aac att gct Asp Glu Gln Asn Val Asn Gly Phe Asn Thr Leu Gly Glu Asn Ile Ala 655 660 665 670	2016
gac aac gga ggg gtg cgg caa gcc tat aag gcc tac ctc aag tgg atg Asp Asn Gly Gly Val Arg Gln Ala Tyr Lys Ala Tyr Leu Lys Trp Met 675 680 685	2064
gca gag ggt ggc aag gac cag cag ctg ccc ggc ctg gat ctc acc cat Ala Glu Gly Gly Lys Asp Gln Gln Leu Pro Gly Leu Asp Leu Thr His 690 695 700	2112
gag cag ctc ttc ttc atc aac tat gcc cag gtg tgg tgc ggg tcc tac Glu Gln Leu Phe Phe Ile Asn Tyr Ala Gln Val Trp Cys Gly Ser Tyr 705 710 715	2160
cgg ccc gag ttc gcc atc caa tcc atc aag aca gac gtc cac agt ccc Arg Pro Glu Phe Ala Ile Gln Ser Ile Lys Thr Asp Val His Ser Pro 720 725 730	2208
ctg aag tac agg gta ctg ggg tcg ctg cag aac ctg gcc gcc ttc gca Leu Lys Tyr Arg Val Leu Gly Ser Leu Gln Asn Leu Ala Ala Phe Ala 735 740 745 750	2256
gac acg ttc cac tgt gcc cgg ggc acc ccc atg cac ccc aag gag cga Asp Thr Phe His Cys Ala Arg Gly Thr Pro Met His Pro Lys Glu Arg 755 760 765	2304
tgc cgc gtg tgg tagccaaggc cctgccgcgc tgtgcccgcc acgcccaccc Cys Arg Val Trp	2356

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770

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gctgctcgga ggcattctgtg cgaaggtgca gctagcggcg acccagtgtg cgtcccggcc 2416
cggccaacca tgccaagcct gcctgccagg cctctgcgcc tggcctaggg tgcagccacc 2476
tgcttgacac ccagggatga gcagtgtcca gtgcagtacc tggaccggag cccccttcac 2536
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<210> SEQ ID NO 15

<211> LENGTH: 770

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 15

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Ala Leu Gly Val Leu Tyr Ala Asp Arg Arg Gly Lys Gln Leu Pro Arg
 35             40             45
Leu Ala Ser Arg Leu Cys Phe Leu Gln Glu Glu Arg Thr Phe Val Lys
 50             55             60
Arg Lys Pro Arg Gly Ile Pro Glu Ala Gln Glu Val Ser Glu Val Cys
 65             70             75             80
Thr Thr Pro Gly Cys Val Ile Ala Ala Ala Arg Ile Leu Gln Asn Met
 85             90             95
Asp Pro Thr Thr Glu Pro Cys Asp Asp Phe Tyr Gln Phe Ala Cys Gly
100             105             110
Gly Trp Leu Arg Arg His Val Ile Pro Glu Thr Asn Ser Arg Tyr Ser
115             120             125
Ile Phe Asp Val Leu Arg Asp Glu Leu Glu Val Ile Leu Lys Ala Val
130             135             140
Leu Glu Asn Ser Thr Ala Lys Asp Arg Pro Ala Val Glu Lys Ala Arg
145             150             155             160
Thr Leu Tyr Arg Ser Cys Met Asn Gln Ser Val Ile Glu Lys Arg Gly
165             170             175
Ser Gln Pro Leu Leu Asp Ile Leu Glu Val Val Gly Gly Trp Pro Val
180             185             190
Ala Met Asp Arg Trp Asn Glu Thr Val Gly Leu Glu Trp Glu Leu Glu
195             200             205
Arg Gln Leu Ala Leu Met Asn Ser Gln Phe Asn Arg Arg Val Leu Ile
210             215             220
Asp Leu Phe Ile Trp Asn Asp Asp Gln Asn Ser Ser Arg His Ile Ile
225             230             235             240
Tyr Ile Asp Gln Pro Thr Leu Gly Met Pro Ser Arg Glu Tyr Tyr Phe
245             250             255
Asn Gly Gly Ser Asn Arg Lys Val Arg Glu Ala Tyr Leu Gln Phe Met
260             265             270
Val Ser Val Ala Thr Leu Leu Arg Glu Asp Ala Asn Leu Pro Arg Asp
275             280             285
Ser Cys Leu Val Gln Glu Asp Met Val Gln Val Leu Glu Leu Glu Thr
290             295             300

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Ala	Leu	Tyr	His	Arg	Met	Gly	Leu	Glu	Glu	Leu	Gln	Ser	Gln	Phe	Gly
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Leu	Lys	Gly	Phe	Asn	Trp	Thr	Leu	Phe	Ile	Gln	Thr	Val	Leu	Ser	Ser
			340					345					350		
Val	Lys	Ile	Lys	Leu	Leu	Pro	Asp	Glu	Glu	Val	Val	Val	Tyr	Gly	Ile
		355					360					365			
Pro	Tyr	Leu	Gln	Asn	Leu	Glu	Asn	Ile	Ile	Asp	Thr	Tyr	Ser	Ala	Arg
	370					375					380				
Thr	Ile	Gln	Asn	Tyr	Leu	Val	Trp	Arg	Leu	Val	Leu	Asp	Arg	Ile	Gly
385					390					395					400
Ser	Leu	Ser	Gln	Arg	Phe	Lys	Asp	Thr	Arg	Val	Asn	Tyr	Arg	Lys	Ala
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Leu	Phe	Gly	Thr	Met	Val	Glu	Glu	Val	Arg	Trp	Arg	Glu	Cys	Val	Gly
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Tyr	Val	Asn	Ser	Asn	Met	Glu	Asn	Ala	Val	Gly	Ser	Leu	Tyr	Val	Arg
		435					440					445			
Glu	Ala	Phe	Pro	Gly	Asp	Ser	Lys	Ser	Met	Val	Arg	Glu	Leu	Ile	Asp
		450				455					460				
Lys	Val	Arg	Thr	Val	Phe	Val	Glu	Thr	Leu	Asp	Glu	Leu	Gly	Trp	Met
465					470					475					480
Asp	Glu	Glu	Ser	Lys	Lys	Lys	Ala	Gln	Glu	Lys	Ala	Met	Ser	Ile	Arg
				485					490					495	
Glu	Gln	Ile	Gly	His	Pro	Asp	Tyr	Ile	Leu	Glu	Glu	Met	Asn	Arg	Arg
			500					505					510		
Leu	Asp	Glu	Glu	Tyr	Ser	Asn	Leu	Asn	Phe	Ser	Glu	Asp	Leu	Tyr	Phe
		515					520					525			
Glu	Asn	Ser	Leu	Gln	Asn	Leu	Lys	Val	Gly	Ala	Gln	Arg	Ser	Leu	Arg
		530				535					540				
Lys	Leu	Arg	Glu	Lys	Val	Asp	Pro	Asn	Leu	Trp	Ile	Ile	Gly	Ala	Ala
545					550					555					560
Val	Val	Asn	Ala	Phe	Tyr	Ser	Pro	Asn	Arg	Asn	Gln	Ile	Val	Phe	Pro
				565					570					575	
Ala	Gly	Ile	Leu	Gln	Pro	Pro	Phe	Phe	Ser	Lys	Glu	Gln	Pro	Gln	Ala
			580					585					590		
Leu	Asn	Phe	Gly	Gly	Ile	Gly	Met	Val	Ile	Gly	His	Glu	Ile	Thr	His
		595					600					605			
Gly	Phe	Asp	Asp	Asn	Gly	Arg	Asn	Phe	Asp	Lys	Asn	Gly	Asn	Met	Met
	610					615					620				
Asp	Trp	Trp	Ser	Asn	Phe	Ser	Thr	Gln	His	Phe	Arg	Glu	Gln	Ser	Glu
625					630					635					640
Cys	Met	Ile	Tyr	Gln	Tyr	Gly	Asn	Tyr	Ser	Trp	Asp	Leu	Ala	Asp	Glu
				645					650					655	
Gln	Asn	Val	Asn	Gly	Phe	Asn	Thr	Leu	Gly	Glu	Asn	Ile	Ala	Asp	Asn
			660					665					670		
Gly	Gly	Val	Arg	Gln	Ala	Tyr	Lys	Ala	Tyr	Leu	Lys	Trp	Met	Ala	Glu
			675				680					685			
Gly	Gly	Lys	Asp	Gln	Gln	Leu	Pro	Gly	Leu	Asp	Leu	Thr	His	Glu	Gln
		690				695					700				
Leu	Phe	Phe	Ile	Asn	Tyr	Ala	Gln	Val	Trp	Cys	Gly	Ser	Tyr	Arg	Pro
705					710					715					720
Glu	Phe	Ala	Ile	Gln	Ser	Ile	Lys	Thr	Asp	Val	His	Ser	Pro	Leu	Lys

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725	730	735	
Tyr Arg Val Leu Gly Ser Leu Gln Asn Leu Ala Ala Phe Ala Asp Thr			
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Phe His Cys Ala Arg Gly Thr Pro Met His Pro Lys Glu Arg Cys Arg			
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Val Trp			
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gcggaggccc acgaggaggc cggcggccac gcgcatcccg tagcccaggt ggcccaggtc			180
tgcaccgcgg cggcctcggc gccg atg gag ccc ccg tat tcg ctg acg gcg			231
Met Glu Pro Pro Tyr Ser Leu Thr Ala			
1 5			
cac tac gat gag ttc caa gag gtc aag tac gtg agc cgc tgc ggc gcg			279
His Tyr Asp Glu Phe Gln Glu Val Lys Tyr Val Ser Arg Cys Gly Ala			
10 15 20 25			
ggg ggc gcg cgc ggg gcc tcc ctg ccc ccg ggc ttc ccg ttg ggc gct			327
Gly Gly Ala Arg Gly Ala Ser Leu Pro Pro Gly Phe Pro Leu Gly Ala			
30 35 40			
gcg cgc agc gcc acc ggg gcc cgg tcc ggg ctg ccg cgc tgg aac cgg			375
Ala Arg Ser Ala Thr Gly Ala Arg Ser Gly Leu Pro Arg Trp Asn Arg			
45 50 55			
cgc gag gtg tgc ctg ctg tcg ggg ctg gtg ttc gcc gcc ggc ctc tgc			423
Arg Glu Val Cys Leu Leu Ser Gly Leu Val Phe Ala Ala Gly Leu Cys			
60 65 70			
gcc att ctg gcg gct atg ctg gcc ctc aag tac ctg ggc ccg gtc gcg			471
Ala Ile Leu Ala Ala Met Leu Ala Leu Lys Tyr Leu Gly Pro Val Ala			
75 80 85			
gcc ggc ggc ggc gcc tgt ccc gag ggc tgc cct gag cgc aag gcc ttc			519
Ala Gly Gly Gly Ala Cys Pro Glu Gly Cys Pro Glu Arg Lys Ala Phe			
90 95 100 105			
gcg cgc gcc gct cgc ttc ctg gcc gcc aac ctg gac gcc agc atc gac			567
Ala Arg Ala Ala Arg Phe Leu Ala Ala Asn Leu Asp Ala Ser Ile Asp			
110 115 120			
cca tgc cag gac ttc tac tcg ttc gcc tgc ggc ggt tgg ctg cgg cgc			615
Pro Cys Gln Asp Phe Tyr Ser Phe Ala Cys Gly Gly Trp Leu Arg Arg			
125 130 135			
cac gcc atc ccc gac gac aag ctc acc tat ggc acc atc gcg gca atc			663
His Ala Ile Pro Asp Asp Lys Leu Thr Tyr Gly Thr Ile Ala Ala Ile			
140 145 150			
ggc gag caa aac gag gag cgc cta cgg cgc ctg ctg gcg cgg ccc ggg			711
Gly Glu Gln Asn Glu Glu Arg Leu Arg Arg Leu Leu Ala Arg Pro Gly			
155 160 165			
ggt ggg cct ggc ggc gcg gcc cag cgc aag gtg cgc gcc ttc ttc cgc			759
Gly Gly Pro Gly Gly Ala Ala Gln Arg Lys Val Arg Ala Phe Phe Arg			
170 175 180 185			
tcg tgc ctc gac atg cgc gag atc gag cga ctg ggc ccg cga ccc atg			807
Ser Cys Leu Asp Met Arg Glu Ile Glu Arg Leu Gly Pro Arg Pro Met			
190 195 200			

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cta gag gtc atc gag gac tgc ggg ggc tgg gac ctg ggc ggc gcg gag Leu Glu Val Ile Glu Asp Cys Gly Gly Trp Asp Leu Gly Gly Ala Glu 205 210 215	855
gag cgt ccg ggg gtc gcg gcg cga tgg gac ctc aac cgg ctg ctg tac Glu Arg Pro Gly Val Ala Ala Arg Trp Asp Leu Asn Arg Leu Leu Tyr 220 225 230	903
aag gcg cag ggc gtg tac agc gcc gcc gcg ctc ttc tcg ctc acg gtc Lys Ala Gln Gly Val Tyr Ser Ala Ala Ala Leu Phe Ser Leu Thr Val 235 240 245	951
agc ctg gac gac agg aac tcc tcg cgc tac gtc atc cgc att gac cag Ser Leu Asp Asp Arg Asn Ser Ser Arg Tyr Val Ile Arg Ile Asp Gln 250 255 260 265	999
gat ggg ctc acc ctg cca gag agg acc ctg tac ctc gct cag gat gag Asp Gly Leu Thr Leu Pro Glu Arg Thr Leu Tyr Leu Ala Gln Asp Glu 270 275 280	1047
gac agt gag aag gtc ctg gca gca tac agg gtg ttc atg gag cga gtg Asp Ser Glu Lys Val Leu Ala Ala Tyr Arg Val Phe Met Glu Arg Val 285 290 295	1095
ctc agc ctc ctg ggt gca gac gct gtg gaa cag aag gcc caa gag atc Leu Ser Leu Leu Gly Ala Asp Ala Val Glu Gln Lys Ala Gln Glu Ile 300 305 310	1143
ctg caa gtg gag cag cag ctg gcc aac atc act gtg tca gag tat gac Leu Gln Val Glu Gln Gln Leu Ala Asn Ile Thr Val Ser Glu Tyr Asp 315 320 325	1191
gac cta cgg cga gat gtc agc tcc atg tac aac aag gtg acg ctg ggg Asp Leu Arg Arg Asp Val Ser Ser Met Tyr Asn Lys Val Thr Leu Gly 330 335 340 345	1239
cag ctg cag aag atc acc ccc cac ttg cgg tgg aag tgg ctg cta gac Gln Leu Gln Lys Ile Thr Pro His Leu Arg Trp Lys Trp Leu Leu Asp 350 355 360	1287
cag atc ttc cag gag gac ttc tca gag gaa gag gag gtg gtg ctg ctg Gln Ile Phe Gln Glu Asp Phe Ser Glu Glu Glu Val Val Leu Leu 365 370 375	1335
gcg aca gac tac atg cag cag gtg tcg cag ctc atc cgc tcc aca ccc Ala Thr Asp Tyr Met Gln Gln Val Ser Gln Leu Ile Arg Ser Thr Pro 380 385 390	1383
cac cgg gtc ctg cac aac tac ctg gtg tgg cgc gtg gtg gtg gtc ctg His Arg Val Leu His Asn Tyr Leu Val Trp Arg Val Val Val Val Leu 395 400 405	1431
agt gaa cac ctg tcc ccg cca ttc cgt gag gca ctg cac gag ctg gca Ser Glu His Leu Ser Pro Pro Phe Arg Glu Ala Leu His Glu Leu Ala 410 415 420 425	1479
cag gag atg gag ggc agc gac aag cca cag gag ctg gcc cgg gtc tgc Gln Glu Met Glu Gly Ser Asp Lys Pro Gln Glu Leu Ala Arg Val Cys 430 435 440	1527
ttg ggc cag gcc aat cgc cac ttt ggc atg gcg ctt ggc gcc ctc ttt Leu Gly Gln Ala Asn Arg His Phe Gly Met Ala Leu Gly Ala Leu Phe 445 450 455	1575
gta cat gag cac ttc tca gct gcc agc aaa gcc aag gtg cag cag cta Val His Glu His Phe Ser Ala Ala Ser Lys Ala Lys Val Gln Gln Leu 460 465 470	1623
gtg gaa gac atc aag tac atc ctg gcc cag cgc ctg gag gag ctg gac Val Glu Asp Ile Lys Tyr Ile Leu Gly Gln Arg Leu Glu Glu Leu Asp 475 480 485	1671
tgg atg gac gcc gag acc agg gct gct gct cgg gcc aag ctc cag tac Trp Met Asp Ala Glu Thr Arg Ala Ala Ala Arg Ala Lys Leu Gln Tyr 490 495 500 505	1719
atg atg gtg atg gtc ggc tac ccg gac ttc ctg ctg aaa ccc gat gct Met Met Val Met Val Gly Tyr Pro Asp Phe Leu Leu Lys Pro Asp Ala	1767

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510	515	520	
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aac atc ttg aac agc atc cgc ttc agc atc cag ctc tca gtt aag aag Asn Ile Leu Asn Ser Ile Arg Phe Ser Ile Gln Leu Ser Val Lys Lys 540	545	550	1863
att cgg cag gag gtg gac aag tcc acg tgg ctg ctc ccc cca cag gcg Ile Arg Gln Glu Val Asp Lys Ser Thr Trp Leu Leu Pro Pro Gln Ala 555	560	565	1911
ctc aat gcc tac tat cta ccc aac aag aac cag atg gtg ttc ccc gcg Leu Asn Ala Tyr Tyr Leu Pro Asn Lys Asn Gln Met Val Phe Pro Ala 570	575	580	1959
ggc atc ctg cag ccc acc ctg tac gac cct gac ttc cca cag tct ctc Gly Ile Leu Gln Pro Thr Leu Tyr Asp Pro Asp Phe Pro Gln Ser Leu 590	595	600	2007
aac tac ggg ggc atc ggc acc atc att gga cat gag ctg acc cac ggc Asn Tyr Gly Gly Ile Gly Thr Ile Ile Gly His Glu Leu Thr His Gly 605	610	615	2055
tac gac gac tgg ggg ggc cag tat gac cgc tca ggg aac ctg ctg cac Tyr Asp Asp Trp Gly Gly Gln Tyr Asp Arg Ser Gly Asn Leu Leu His 620	625	630	2103
tgg tgg acg gag gcc tcc tac agc cgc ttc ctg cga aag gct gag tgc Trp Trp Thr Glu Ala Ser Tyr Ser Arg Phe Leu Arg Lys Ala Glu Cys 635	640	645	2151
atc gtc cgt ctc tat gac aac ttc act gtc tac aac cag cgg gtg aac Ile Val Arg Leu Tyr Asp Asn Phe Thr Val Tyr Asn Gln Arg Val Asn 650	655	660	2199
ggg aaa cac acg ctt ggg gag aac atc gca gat atg ggc ggc ctc aag Gly Lys His Thr Leu Gly Glu Asn Ile Ala Asp Met Gly Gly Leu Lys 670	675	680	2247
ctg gcc tac cac gcc tat cag aag tgg gtg cgg gag cac ggc cca gag Leu Ala Tyr His Ala Tyr Gln Lys Trp Val Arg Glu His Gly Pro Glu 685	690	695	2295
cac cca ctt ccc cgg ctc aag tac aca cat gac cag ctc ttc ttc att His Pro Leu Pro Arg Leu Lys Tyr Thr His Asp Gln Leu Phe Phe Ile 700	705	710	2343
gcc ttt gcc cag aac tgg tgc atc aag cgg cgg tcg cag tcc atc tac Ala Phe Ala Gln Asn Trp Cys Ile Lys Arg Arg Ser Gln Ser Ile Tyr 715	720	725	2391
ctg cag gtg ctg act gac aag cat gcc cct gag cac tac agg gtg ctg Leu Gln Val Leu Thr Asp Lys His Ala Pro Glu His Tyr Arg Val Leu 730	735	740	2439
ggc agt gtg tcc cag ttt gag gag ttt ggc cgg gtt tta cac tgt cca Gly Ser Val Ser Gln Phe Glu Glu Phe Gly Arg Val Leu His Cys Pro 750	755	760	2487
aag gtc tca ccc atg aac cct gcc cac aag tgt tcc gtg tgg Lys Val Ser Pro Met Asn Pro Ala His Lys Cys Ser Val Trp 765	770	775	2529
tgaccctggc tgcccgcctg cacgccccca ctgccccgc acgaatcacc tccctgctggc			2589
taccggggca ggcattgcacc cgggtgccagc cccgctctgg gcaccacctg ccttccagcc			2649
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gctggacttt ggggggctgt gagggaaata tactggggtc cccagattct gctctaaggg			2769
ggccagacc tctgccagc tggattgtac gggccccacc ttcgctgtgt tcttgctgca			2829
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<210> SEQ ID NO 17
<211> LENGTH: 775
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

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Val Lys Tyr Val Ser Arg Cys Gly Ala Gly Gly Ala Arg Gly Ala Ser
 20          25          30
Leu Pro Pro Gly Phe Pro Leu Gly Ala Ala Arg Ser Ala Thr Gly Ala
 35          40          45
Arg Ser Gly Leu Pro Arg Trp Asn Arg Arg Glu Val Cys Leu Leu Ser
 50          55          60
Gly Leu Val Phe Ala Ala Gly Leu Cys Ala Ile Leu Ala Ala Met Leu
 65          70          75          80
Ala Leu Lys Tyr Leu Gly Pro Val Ala Ala Gly Gly Gly Ala Cys Pro
 85          90          95
Glu Gly Cys Pro Glu Arg Lys Ala Phe Ala Arg Ala Ala Arg Phe Leu
100          105          110
Ala Ala Asn Leu Asp Ala Ser Ile Asp Pro Cys Gln Asp Phe Tyr Ser
115          120          125
Phe Ala Cys Gly Gly Trp Leu Arg Arg His Ala Ile Pro Asp Asp Lys
130          135          140
Leu Thr Tyr Gly Thr Ile Ala Ala Ile Gly Glu Gln Asn Glu Glu Arg
145          150          155          160
Leu Arg Arg Leu Leu Ala Arg Pro Gly Gly Gly Pro Gly Gly Ala Ala
165          170          175
Gln Arg Lys Val Arg Ala Phe Phe Arg Ser Cys Leu Asp Met Arg Glu
180          185          190
Ile Glu Arg Leu Gly Pro Arg Pro Met Leu Glu Val Ile Glu Asp Cys
195          200          205
Gly Gly Trp Asp Leu Gly Gly Ala Glu Glu Arg Pro Gly Val Ala Ala
210          215          220
Arg Trp Asp Leu Asn Arg Leu Leu Tyr Lys Ala Gln Gly Val Tyr Ser
225          230          235          240
Ala Ala Ala Leu Phe Ser Leu Thr Val Ser Leu Asp Asp Arg Asn Ser
245          250          255
Ser Arg Tyr Val Ile Arg Ile Asp Gln Asp Gly Leu Thr Leu Pro Glu
260          265          270
Arg Thr Leu Tyr Leu Ala Gln Asp Glu Asp Ser Glu Lys Val Leu Ala
275          280          285
Ala Tyr Arg Val Phe Met Glu Arg Val Leu Ser Leu Leu Gly Ala Asp
290          295          300
Ala Val Glu Gln Lys Ala Gln Glu Ile Leu Gln Val Glu Gln Gln Leu
305          310          315          320
Ala Asn Ile Thr Val Ser Glu Tyr Asp Asp Leu Arg Arg Asp Val Ser
325          330          335
Ser Met Tyr Asn Lys Val Thr Leu Gly Gln Leu Gln Lys Ile Thr Pro
340          345          350
His Leu Arg Trp Lys Trp Leu Leu Asp Gln Ile Phe Gln Glu Asp Phe
355          360          365
Ser Glu Glu Glu Glu Val Val Leu Leu Ala Thr Asp Tyr Met Gln Gln
370          375          380

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What is claimed is:

1. An isolated nucleic acid comprising a nucleotide sequence encoding a metallopeptidase having at least about 95% amino acid sequence identity with the complete amino acid sequence of SEQ ID NO: 13.

2. A recombinant vector comprising the isolated nucleotide sequence of claim 1.

3. An isolated host cell transformed with the vector of claim 2.

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4. An isolated nucleic acid comprising the nucleotide sequence of SEQ ID NO: 12.

5. A recombinant vector comprising the isolated nucleotide sequence of claim 4.

6. An isolated host cell transformed with the vector of claim 5.

* * * * *